creative commons
COMMONS DEED

저작자표시-비영리-변경금지 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.

변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

A THESIS FOR THE DEGREE OF

MASTER OF SCIENCE IN FOOD AND NUTRITION

Effects of Mild Calorie Restriction on the Markers Related to Lipid Metabolism and Inflammation in Liver and Adipose Tissue

경정도의 열량제한이 간과 지방조직의
지질대사와 염증반응에 미치는 영향

February, 2014

Department of Food and Nutrition

Graduate School

Seoul National University

Chan Yoon Park

경정도의 열량제한이 간과 지방조직의 지질대사와 염증반응에 미치는 영향

Effects of Mild Calorie Restriction on the Markers Related to Lipid Metabolism and Inflammation in Liver and Adipose Tissue

지도교수 한 성 림

이 논문을 생활과학석사 학위논문으로 제출함

2013년 11월

서울대학교 대학원
식품영양학과
박 찬 윤

박찬윤의 생활과학 석사학위 논문을 인준함
2014년 1월

위 원 장 _____ (인)

부 위 원 장 _____ (인)

위 원 _____ (인)

Abstract

Effects of Mild Calorie Restriction on the Markers Related to Lipid Metabolism and Inflammation in Liver and Adipose tissue

Chan Yoon Park

Department of Food and Nutrition

The Graduate School

Seoul National University

The incidence of obesity and disorders induced by obesity is increasing worldwide. With obesity, the excess energy storage leads to the expansion of adipocyte and the number of macrophages in white adipose tissue increases, which results in release of pro-inflammatory cytokines from the adipose tissue. Calorie restriction (CR) has been reported to decrease adiposity and inflammation. However, most CR models use 30~60% of calorie reduction, which is hard to achieve in human. We investigated the effects of mild CR on hepatic lipid metabolism and inflammation. Seven-week-old C57BL/6 mice were fed control diet containing 10% kcal fat (Control) or high fat diet containing 60% kcal fat (HFD) or reduced amount of control diet to achieve 14.1% calorie restriction (CR). We measured body weight, food intake, liver weight, hepatic lipid contents, serum levels of triglyceride (TG), cholesterol,

non-esterified fatty acid (NEFA), alanine transaminase (ALT), aspartate transaminase (AST), glucose, leptin, adiponectin, and fetuin-A. The mRNA levels of hepatic and epididymal *IL-1β, IL-6, Mcp-1, Tnf-α,* and *Socs3*, hepatic *Pparg, Ppara, Fasn, Srebf-1c, Adipor2,* and *Cpt1a* and protein level of hepatic SIRT1 and pSTAT3/STAT3 were determined. CR group had significantly lower body weight gain, adipose tissue amount, liver weight, and liver TG, and cholesterol levels than the Control and HFD groups. Serum level of TG was significantly lower in CR group than HFD group ($P = 0.024$) and serum levels of cholesterol and glucose were significantly lower in CR group than Control and HFD groups. Hepatic expression of genes related to lipogenesis, *Pparg* and *Srebf-1c,* were significantly lower in CR and Control groups compared with HFD group. In epididymal adipose tissue, CR group showed significantly lower mRNA expression of pro-inflammatory chemokine, *Mcp-1*, compared with other two groups. The mRNA expression of epididymal *IL-6* was significantly lower in CR group than HFD group ($P < 0.001$) and tended to be lower in CR group, compared with Control group ($P = 0.057$). Also, the mRNA levels of epididymal *IL-1β, Tnf-α,* and *Socs3* in CR group were significantly lower than HFD group, but not significantly lower than Control group. In liver tissue, *Mcp-1* mRNA level was lower in CR group than Control and HFD groups (63% and 74% lower, respectively). Hepatic *Tnf-α* mRNA level was lower in CR group

than HFD group (47% lower). However, mRNA levels of *IL-6* and *Socs3* were significantly higher in CR group than HFD group (*IL-6*, P = 0.013; *Socs3*, P = 0.009). Also, the hepatic *Lepr* mRNA level was significantly higher in CR group than Control and HFD groups (P = 0.001 and, P < 0.001, respectively). Hepatic *IL-6* mRNA level was correlated positively with hepatic *Socs3* (r = 0.446, P = 0.049), and pSTAT3/STAT3 ratio (r = 0.476, P = 0.034), and tended to correlate negatively with serum glucose (r = -0.419, P = 0.066). Also, hepatic expression of *Lepr* showed correlations with hepatic *Socs3* (r = 0.699, P< 0.001), and serum glucose level (r = -0.717, P < 0.001), and tended to have positive correlation with pSTAT3/STAT3 ratio (r = 0.432, P = 0.051). It suggested that low level of serum glucose in CR group increased hepatic *IL-6* expression and leptin sensitivity, resulted in up-regulated *Socs3* expression through JAK-STAT3 signaling to spare glucose. In conclusion, mild CR group reduced body weight gain, and hepatic lipid levels and alleviated inflammatory responses in adipose and liver tissues. Also CR might have caused beneficial hepatic insulin resistance to prevent consumption of glucose in the liver.

KEY WORDS: Mild calorie restriction, Hepatic inflammation, Adipose tissue inflammation, Hepatic lipid metabolism, Socs3 regulation, Leptin sensitivity

Student Number: 2012-21491

Contents

List of Tables

List of Figures

List of Abbreviations

ACC, acetyl-CoA carboxylase

ALT, alanine transaminase

AST, aspartate transaminase

AdipoR2, adiponectin receptor2

CCL2, chemokine ligand 2

CNS, central nervous system

CPT1, carnitine palmitoyltransferase 1

CR, calorie restriction

ERK, extracellular signal-regulated kinase

FASN, fatty acid synthase

FABP1, fatty acid binding protein1

FFA, free fatty acid

FOXO, forkheadbox protein O

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

IL-1β, interleukin 1β

IL-6, interleukin 6

IKK, I kappa B kinase

IRS-1, insulin receptor substrate 1

JNK, c-Jun N-terminal kinase

LCFA, long chain fatty acid

LEPR, leptin receptor

LXRα, liver X receptor alpha

MCP-1, monocyte chemoattractant protein 1

NAFLD, non-alcoholic fatty liver disease

NASH, non-alcoholic steatohepatitis

NEFA, non-esterified fatty acid

NF-κB, nuclear factor kappa-light chain-enhancer of activated B cells

PPARg, peroxisome proliferator-activated receptor gamma

PPARa, peroxisome proliferator-activated receptor alpha

ROS, reactive oxygen species

SIRT1, NAD-dependent deacetylase sirtuin-1

SREBP-1C, sterol regulatory element-binding protein

SOCS3, suppressor of cytokine signaling 3

STAT3, signal transducer and activator of transcription 3

TNF-α, tumor necrosis factor alpha

TLR, toll like receptor

Ⅰ. Introduction

The incidence of obesity and disorders induced by obesity is increasing worldwide. Obesity is a serious health issue in westernized countries and it has been affecting even developing countries. Failure of homeostatic mechanisms regulating fat storage and energy utilization leads to obesity (Kaplan 1998). Excess energy storage in adipocyte leads to the expansion of adipose tissue and alters pathologic processes including immunity and inflammation. (Fantuzzi 2005). Therefore, obesity could increase the risk of chronic disorders such as insulin resistance and non-alcoholic fatty liver disease (NAFLD). These complications of obesity occur not only with simple accumulation but also with chronic inflammation (Hotamisligil et al. 1993).

Adipose tissue is no longer considered as a simple site of energy storage, rather it is considered as a metabolically active site and a releasing site that releases various products known as adipocytokines such as adiponectin, and leptin, and pro-inflammatory molecules. As the size of adipocyte expands, the number of macrophages in white adipose tissue is increasing (Fantuzzi 2005, Weisberg et al. 2003, Xu et al. 2003). Monocyte chemoattractant protein-1 (Mcp-1), released from adipocyte, could contribute to macrophage infiltration into adipose tissue (Kanda et al. 2006, Weisberg et al. 2006). In obese state, not only the number of macrophage is increasing, but the

phenotype of macrophage is changing from M2(anti-inflammatory) to M1(pro-inflammatory). Both infiltrated macrophages with an M1 phenotype and expanded adipocyte produce pro-inflammatory cytokines, resulting in activation of other inflammatory pathways. Meanwhile, in the liver tissue, instead of macrophages infiltration during obesity, macrophage-like kupffer cell is activated and releases also pro-inflammatory cytokines. As a result, obesity leads to chronic inflammation in both adipose and liver tissues.

Calorie restriction has been reported to reduce inflammation as well as improve disorders related to obesity. Calorie restriction in rodents increased adiponectin level and decreased hepatic lipid peroxidation and cytokine expression in adipose tissue, which resulted in attenuated inflammatory responses (Huang et al. 2010, Park et al. 2012). Also weight loss through low calorie intake of obese women reduced circulating Interleukin-6(IL-6) concentration (Bastard et al. 2000). Consequentially, these anti-inflammatory effects of calorie restriction are associated with longevity (Yu 2006). Life extension and anti-inflammatory effects induced by calorie restriction might be mediated by elevated SIRT1 protein expression (Cohen et al. 2004, Tauriainen et al. 2011).

Although benefits of calorie restriction have been already reported in many animal studies, these benefits would be hard to achieve in human as more than 30% of calorie restrictions were used for traditional mammalian models

of calorie restriction. It is unrealistic for most humans to practice these severe degrees of calorie restriction in real life (Cohen et al. 2004, Huang et al. 2010, Li et al. 2010). Therefore, in the current study we investigated the effects of mild calorie restriction on lipid metabolism and inflammation in liver and adipose tissue.

Objective of our study was to investigate the effect of mild calorie restriction with 14.1% calorie reduction from control diet (10% kcal fat) on hepatic lipid oxidation, lipogenesis, and chronic inflammation in C57BL/6 mice. The response to mild calorie restriction was compared with the control group as well as high fat diet-induced obese group.

Ⅱ. Literature review

1. Diet-induced obesity and lipid metabolism in liver

1-1. Non-alcoholic fatty liver disease (NAFLD)

Obesity is caused by increased energy intake and decreased energy expenditure that results in increased adipose tissue (Gregor et al. 2011). The world health organization estimates that more than one billion adults are overweight, 300 million of whom are clinically obese defined as ‘BMI equal or higher than 30 (Hotamisligil 2006). Obesity, especially induced by high fat diet, is generally considered that it can induce many chronic disorders such as insulin resistance, type 2 diabetes, and non-alcoholic fatty liver diseases (NAFLD) (Carmiel-Haggai et al. 2005, Gregor et al. 2011, Kahn et al. 2006). NAFLD, which is characterized by increased adiposity in the liver, comprises a wide spectrum of liver damage, ranging from steatosis to nonalcoholic steatohepatitis (NASH) with fibrosis (Alba et al. 2003).

Generally, NAFLD is explained by ‘two-hit hypothesis’. First hit is caused by simple steatosis and second hit is followed by inflammatory mediators such as pro-inflammatory cytokines and reactive oxygen species (ROS) (Musso et al. 2009). Nonalcoholic steatosis is related to mitochondrial abnormality, which could increase activity of cytochrome P-450 system and generation of free oxygen radicals. Thus simple steatosis has the potential to progress through the inflammatory phase of NASH. Also, beta oxidation

within the peroxisome and omega oxidation with in the ER, are producing hepatic free fatty acids (FFAs), resulted in ROS formation and Tnf receptor activation. And up-regulated hepatic FFA also increased expression of Srebp-1c and facilitated activation of the extrinsic apoptosis cascade (Bechmann et al. 2012). Injured liver produces cytokine signals that lead to adipose tissue to release FAs into the circulation, to fibrosis, cirrhosis (20%), and in some case (9%) to liver failure or hepatic cellular carcinoma (HCC) (1%) (Liu et al. 2010, Valdecantos et al. 2012).

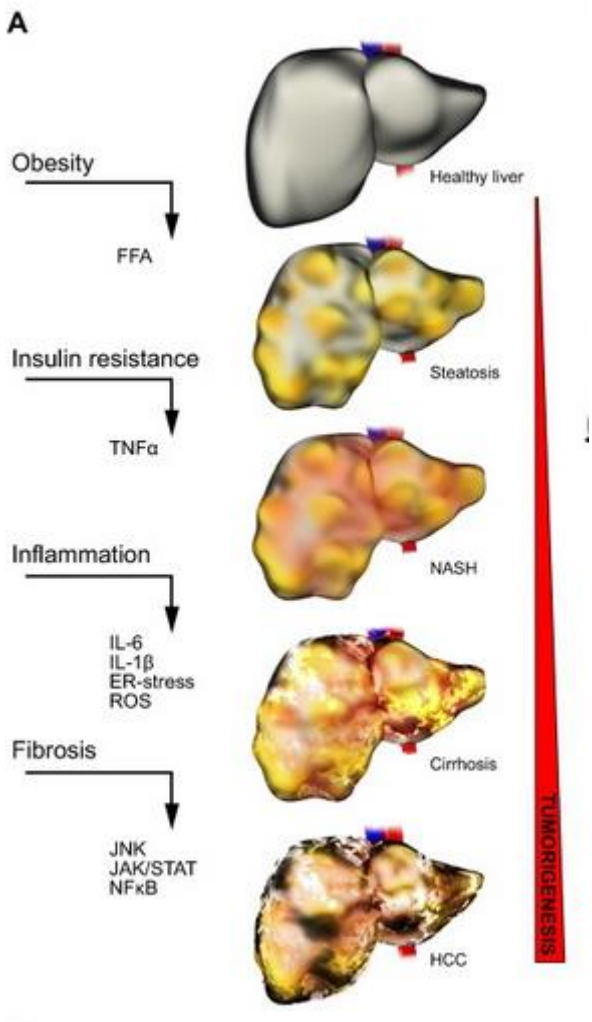


Figure 1. Mediators of NAFLD progression (Bechmann et al. 2012)

1-2. Hepatic lipid accumulation mechanism

Hepatic lipid accumulation is resulted from an imbalance between lipid availability and lipid disposal. Lipid availability is regulated by FFA input via dietary FA uptake or de novo lipogenesis. And lipid disposal is regulated by FFA output via fatty acid oxidation or lipoprotein secretion (Fabbrini et al. 2010, Musso et al. 2009).

FAs in the liver, mainly through the fatty acid transport proteins, is come from the peripheral fat stored in adipose tissue or dietary fat by the way of the plasma NEFA pool (Musso et al. 2009). Usually obese subjects with NAFLD showed high expression of hepatic lipoprotein lipase, suggesting that FFA released from lipolysis of circulating TG contributed to hepatocellular FFA accumulation (Fabbrini et al. 2010, Pardina et al. 2009). Also they showed high expression of hepatic membrane protein that contributed to transport FFA into liver.

De novo lipogenesis is comprised of many metabolic pathways, in which acetyl CoA is converted to malonyl-CoA by acetyl CoA carboxylase. Malonyl-CoAs undergo several pathways to synthesize fatty acid and finally they form TG in the liver (Fabbrini et al. 2010, Koo 2013). Lipogenesis pathways have rate limiting enzymes, acetyl CoA carboxylase (Acc) and fatty acid synthase (Fasn) involved in lipogenesis. And there are transcription factors, sterol regulatory element binding protein 1c (Srebp-1c)

and carbohydrate response element binding protein (ChREBP), contributing to activation of genes encoding aforementioned rate limiting enzymes. Fatty liver of the high fat diet induced mice model showed high expression of SREBP-1c and SREBP-1c transgenic mice showed high expression of hepatic lipogenic gene as well (Shimomura et al. 1999). Also, ChREBP knock-out mice showed reduced Fasn and Acc1 mRNA expression level, suggesting that deficiency of ChREBP reduces lipogenesis (Iizuka et al. 2004).

Liver is one of the most energy requiring tissue, and hepatic energy is provided from fatty acid oxidation and amino acid oxidation. The beta oxidation of intra-hepatocellular fatty acid occurs with in mitochondria, and it is the process to shorten the fatty acid to produce ATP through TCA cycle. Transport of long chain fatty acid into the mitochondria is regulated by carnitine palmitoyl transferase1&2 (Cpt1, Cpt2), carnitine acylcarnitine translocase (Cat) (Fabbrini et al. 2010, Koo 2013). Through fatty acid oxidation, amounts of accumulated TGs and hepatic FFAs are decreased in the liver tissue.

Lastly, liver regulates hepatic lipid through TG secretion by the formation of very low density lipoprotein (VLDL) (Koo 2013). VLDL is consisted of TGs, cholesterols, cholesterol esters and VLDL is covered with hydrophilic phospholipids and apo-lipoprotein B 100 (apoB100). In non-obese state, rate

of TG secretion is increasing linearly with intrahepatic TG contents. However, if TG infiltration into liver exceeded 10% in NAFLD, TG secretion through VLDL is limited and lipids are accumulated in the liver (Musso et al. 2009)

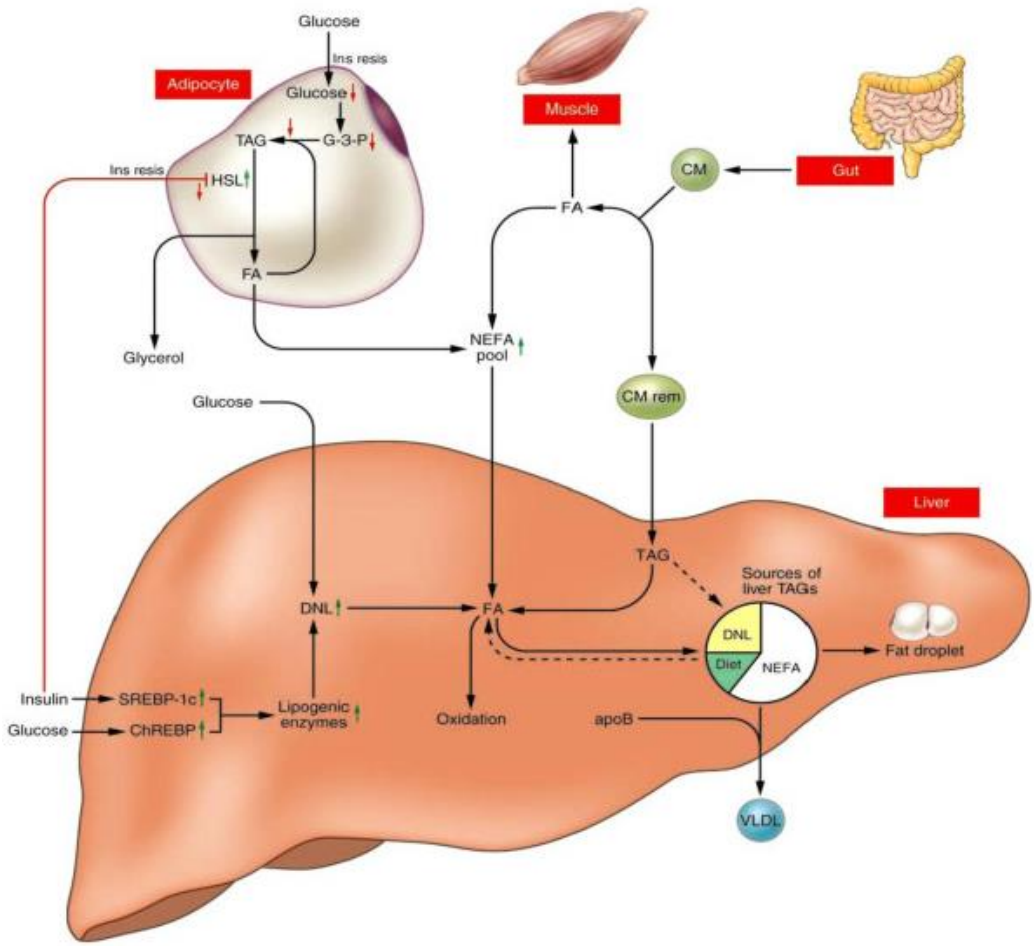


Figure 2. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease (Liu et al. 2010)

1-3. Genes related to hepatic lipid oxidation and lipid synthesis

Generally, high fat diet-induced obesity inhibited the expression of genes related to hepatic fatty acid oxidation and promoted genes related to lipid synthesis in the liver tissue.

1) Carnitine palmitoyl transferase1 (Cpt1)

Carnitine palmitoyl transferase1 (Cpt1) is an integral mitochondrial outer membrane protein. For energy production, long chain fatty acids transfer into the mitochondrial double membrane, through cpt1. Malonyl CoA, contributing to fatty acid synthesis, is the inhibitor of the cpt1a. Malonyl CoA, found in variety tissue such as adipocyte, skeletal muscle and liver, allosterically binds to Cpt1 with reducing the transfer of long chain fatty acids into the mitochondria (Gobin et al. 2003). Cpt1 is up-regulated by Ppara, contributing to the reduction of plasma free fatty acids (Bragt et al. 2008).

2) Peroxisome proliferator- activated receptor alpha (Ppara)

Peroxisome proliferator- activated receptor alpha (Ppara) is the isoform of the Ppar subclass of the nuclear hormone receptor superfamily, activated by the peroxisome proliferator chemicals (Bragt et al. 2008). Mainly, Ppara is abundant in liver, heart, and skeletal muscle where Ppara could function as fatty acid homeostasis regulator (Desvergne et al. 1999) Ppara, which is stimulated by plasma FFA level, enhances fatty acid catabolism through up-

regulating transcriptional expression of genes, such as Fabp, Cpt1, Cpt2, and acyl CoA synthase. Also, Ppara controls the lipoprotein metabolism, by increasing lipoprotein lipase, thereby decreasing LDL cholesterol and TG level, and by increasing HDL cholesterol level, stimulating cholesterol efflux from peripheral tissue (Azhar 2010, Bragt et al. 2008, Desvergne et al. 1999).

3) Sterol regulatory element-binding protein-1c (Srebp-1c)

Srebp-1c, as one of the SREBP family, is the transcription factor of genes related to cellular availability in cholesterol. Srebp-1c is expressed in most tissue of mice and human with especially high levels in the liver (Musso et al. 2009). Srebp-1c expression is regulated by hormone, other genes, and nutritional status. Insulin treatment markedly increases Srebp-1c expression by the phosphoinositide 3-kinase-dependent pathway. And LXRα, one of the activator of Srebp-1c transcription, induces Srebp-1c in order to synthesize fatty acids needed for the formation of cholesterol esters (Ferre et al. 2010).

4) Fatty acid synthase (Fasn)

Fatty acid synthase (Fasn) catalyzes the last step of the saturated fatty acid biosynthesis from simple precursors. The primary product of the Fasn reaction is palmitate and substrates of the Fasn are acetyl-CoA, malonyl-CoA, and NADPH (Jensen-Urstad et al. 2012). Lipids synthesized by the Fasn are mainly stored as lipid droplets in the liver tissue or other tissue.

However surprisingly, Fasn knockout mice did not be protected against hepatic steatosis but rather developed fatty liver with normal diet (Chakravarthy et al. 2005). This result suggested that newly fatty acid, derived from de novo lipogenesis through Fasn activation, can activate Ppara in the liver (Chakravarthy et al. 2005, Musso et al. 2009). Fasn seems to contribute to Ppara activity by promoting the synthesis of one of its ligands (Jensen-Urstad et al. 2012). Also, Srebp-1c is one of the transcription factors of the Fasn, resulted in promoted Fasn expression by insulin (Foretz et al. 1999).

5) Peroxisome proliferator- activated receptor gamma (Pparg)

Peroxisome proliferator- activated receptor gamma (Pparg) is one of the group of lipid sensing ligand activated by transcriptional factors and it is expressed in the adipose tissue (Mathieu et al. 2010). Pparg, involved in adipocyte differentiation and lipid storage, reduces free fatty acid by increasing fatty acid uptake and storage and by increasing mRNA expression of CD36, a gene belonging to the class B scavenger receptor superfamily. Pparg also regulates glucose homeostasis by activating GLUT4, glucokinase, and phosphoenolpyruvate carboxylase. Therefore as the Pparg agonist, pioglitazone decreases plasma triglycerides and rosiglitazone increases LDL particles (Desvergne et al. 1999, Mathieu et al. 2010).

Pparg also has anti-inflammatory effects by regulating insulin signaling

and the pro-inflammatory cytokines such as Tnf-α, IL-6. Pparg inactivate transcription factors, such as NF-κB, and AP1 and interact with STAT, thereby reducing expression of the pro-inflammatory cytokines (Azhar 2010, Desvergne et al. 1999).

2. Obesity and inflammatory response

Classical inflammation is described as principal responses of the body to resist with pathogen from external body, defined by the sign of swelling, redness, pain and fever (Gregor et al. 2011, Hotamisligil 2006). It is usually short term adaptive response and beneficial to body. However, obesity induced inflammation is chronic response and is related to other metabolic diseases such as insulin resistance and cardiovascular diseases (CVD).

Obesity triggers inflammation by macrophage infiltration through accumulation of adipose tissue, excess fatty acid oxidation and abnormal immune sensor response. First, in the high fat diet(HFD)-induced obesity model, expanded adipocyte and accumulated macrophage secrete inflammatory cytokines(Buckman et al. 2013, Nteeba et al. 2013). Nteeba et al. showed increased adipocyte size, morphological presentation of infiltrating immune cells at adipose tissue, and activated macrophage in HFD induced mice. Also HFD-induced obese rats showed recruitment of peripheral immune cells into the central nervous system (CNS). Second, excess intracellular fatty acid can induce oxidative stress by increased beta oxidation resulted in producing reactive oxygen species (ROS). In the hepatocyte, ROS can activate protein kinase such as extracellular signal-regulated kinase 1/2(ERK1/2), c-Jun N-terminal kinase (JNK), and the nuclear factor kappa B (NF-κB) pathway. (Gregor et al. 2011, Maher et al.

2008) Third, immune sensors known as Toll-like receptors (TLRs) are more activated in obese tissue compared with lean controls (Gregor et al. 2011). TLRs are cellular pattern recognizing receptors (PPRs) that response to pattern of pathogens such as endotoxin. (Liu et al. 2010) Song and colleagues showed that in 3T3-L1 adipocyte, free fatty acids in common with LPS, stimulated NF-κB signaling and cytokine secretion, suggesting that obesity and type 2 diabetes might stimulate the activation of TLR4 in adipocyte. (Song et al. 2006)

2-1. Inflammation in adipose tissue

Adipose tissue is the hormonally active system involved in insulin action as well as glucose and fatty acid metabolism. A variety of adipocyte-derived soluble factors are known as adipocytokines that interact relationship between adipose tissue, inflammation and Immunity (Tilg et al. 2006, Xydakis et al. 2004). Adiponectin and leptin are the most abundant adipocytokines. Mcp-1, IL-6, Tnf-α and other cytokines are secreted from the macrophage in adipocyte and secreted from adipocyte itself.

2-2. Pro-inflammatory cytokines and chemokine

1) Chemokine: Monocyte chemoattractant 1(Mcp-1)

Monocyte chemoattractant protein-1 is produced mostly by macrophage and endothelial cells and is a kind of chemokine. Expression of Mcp-1 is increased in atherosclerotic lesions. Chemokines recruit monocytes into the

sub-endothelium of damaged arteries. Recruitment of blood monocytes into the arterial sub-endothelium is one of the earliest steps in atherogenesis (Kanda et al. 2006). LDL-receptor deficient mice with Mcp-1 deficient had 83% less lipid deposition throughout their aortas compared with LDL-receptor deficient mice with wild type Mcp-1 alleles (Gu et al. 1998). Also Mcp-1 is a kind of adipokine whose expression is up-regulated in obesity. Thus, Mcp-1 produced by expanded adipocyte contributes to macrophage infiltration into adipose tissue (Tilg et al. 2006). As the ligand of Mcp-1, C-C motif chemokine receptor (Ccr2), Ccr2 contributes to the regulation of adipocyte function as well. In obese mice with Ccr2 deficiency reduced macrophage content and inflammatory cytokines, also obese mice with a pharmacological antagonist of Ccr2 lowered macrophage content in adipose tissue(Weisberg et al. 2006). Therefore both Mcp-1 and its receptor Ccr2 are needed to regulate adipocyte inflammation.

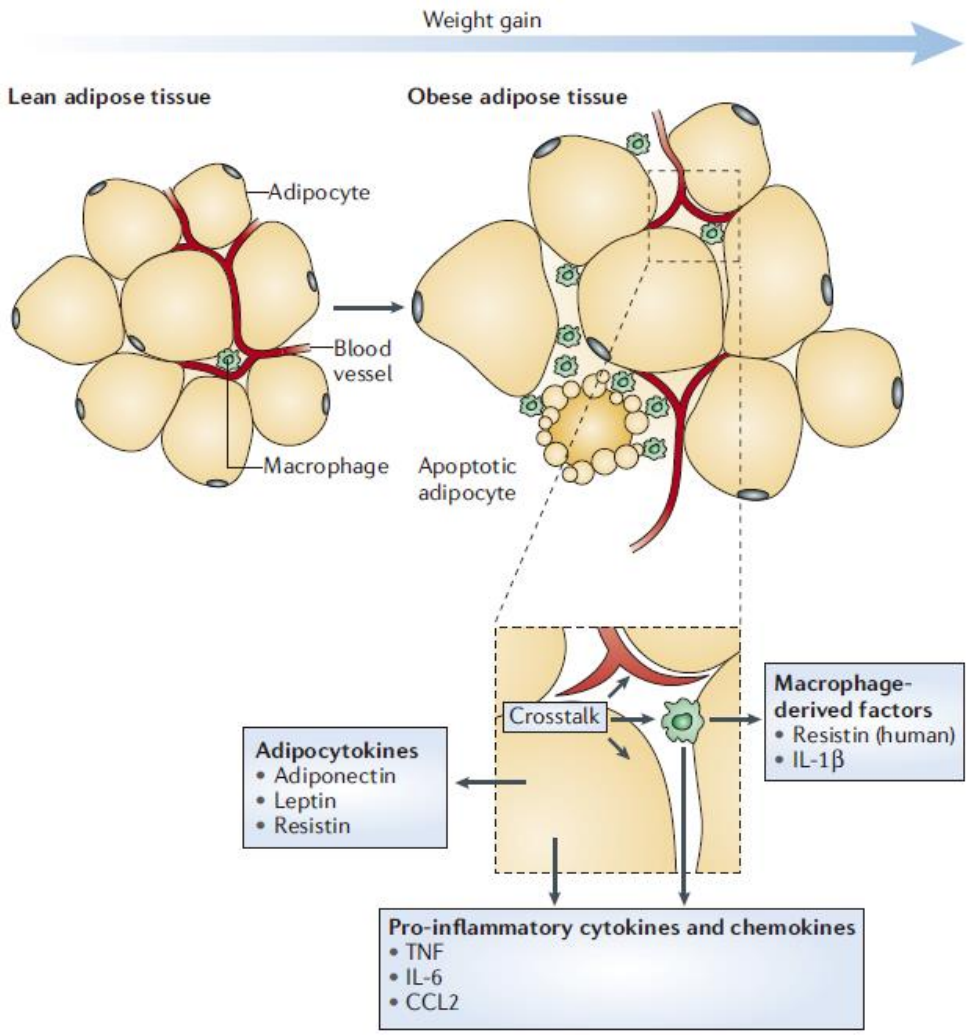


Figure 3. Adipose tissue: cellular components and molecule synthesized (Tilg et al. 2006)

2) Cytokine: Interleukin 6 (IL-6), Tumor necrosis factor α (Tnf-α)

IL-6 and Tnf-α are the adipocytokines produced in the adipose tissue. Therefore they are increased on the serum and white adipose tissue of obese subjects.

Tnf-α is associated with obesity and insulin resistance (Hotamisligil et al. 1993). Insulin sensitivity of Tnf-α knock-out mice and Tnf-α receptor knock-out mice was improved, since Tnf-α inhibits phosphorylation of serine residue of IRS1 (Aguirre et al. 2000). Tnf-α was expressed not only in adipose tissue but also expressed in liver of the obese subjects. However, Tnf-α expression was 5.7 fold higher and 4.3 fold higher in subcutaneous and visceral adipose tissue, compared with hepatic expression (Gerner et al. 2013). It remains unclear whether hepatic Tnf-α is mainly responsible for hepatic insulin resistance, even though hepatic Tnf-α has been identified as contributed to hepatic inflammation (Gerner et al. 2013, Tilg et al. 2000). IL-6 is also mainly produced in adipose tissue and IL-6 contributed to insulin resistance. IL-6 is very sensitive pro-inflammatory cytokine expressed in adipocyte, because its expression is increasing or decreasing linearly with fat amount (Gerner et al. 2013). Both adipocytes and macrophage contribute to white adipose tissue derived IL-6 (Tilg et al. 2006).

2-3. Inflammation in liver

Inflammatory responses in liver are different from those in adipose tissue. In contrast to adipose tissue, the liver did not let macrophage infiltrate into liver during the obese state. Instead of macrophage infiltration, there is resident macrophage-like kupffer cell within the cells of the liver (Gregor et al. 2011). In animal models of obesity, kupffer cells secreted more inflammatory cytokines than lean controls (Cai et al. 2005).

In obese liver, inflammatory mediators have the ability to inhibit insulin signaling through activation of NF-κB. Excess fat activated JNK and IKK in hepatocytes, which can induce hepatic insulin resistance, inflammatory cytokine expression. JNK and IKK both have the ability to stimulate the transcription of inflammatory target genes through their activation of activator protein-1(AP-1) and NF-κB, respectively (Arkan et al. 2005, Cai et al. 2005, Maher et al. 2008). High-fat diet induced obesity triggered expression of the hepatic inflammatory gene closely mimicking the hepatocyte-specific IKK transgene mice (Arkan et al. 2005, Maher et al. 2008). Also inflammatory related genes lead to lipogenesis in liver. Absence of JNK in liver resulted in decreased adiposity. And hepatic IL-6, TNF-α could induce hepatic lipogenesis and increase hepatic TG production as well (Grunfeld et al. 1990, Hirosumi et al. 2002). Liver is not major source of cytokines in obese state and it induces less cytokines relative to adipose

tissue which produces 100 to 1000 times more of various pro-inflammatory cytokines such as Tnf-α, IL-1β, IL-6 (Gerner et al. 2013). Therefore high secretion of cytokine from adipose tissue could affect liver inflammatory diseases.

2-4. Suppressor of cytokine signaling 3 (Socs3) signaling

Suppressor of the cytokine signaling (Socs3) family is the negative regulator of cytokine signaling, especially inhibitor of the JAK-STAT signal transduction (Palanivel et al. 2012). Not only negatively regulate cytokine signaling, but also Socs family is induced by the cytokine signaling. Among them, Socs3, one of the member of Socs family, binds to JAK kinases and inhibits several cytokine receptor signaling pathways by binding to phosphotyrosine residues of receptors (Senn et al. 2003). Also Socs3 expression is induced by leptin signaling as well as cytokines, such as IL-6 and Tnf-α and up-regulated Socs3 expression blocked leptin receptor (Bjorbaek et al. 1999, Emanuelli et al. 2001) **(Figure4).** Generally, cytokine and leptin secretions are increasing in obese state, therefore up-regulated Socs3 expression is also found in the adipose tissue and hypothalamus of obese animals. Although Socs3 expression is also increased in liver of the db/ db mice, it is uncertain that whether high-fat diet induced obesity also increased the expression of the Socs3 in the liver (Ueki et al. 2004). Because

unlike the study of Ueki et al (2004), Kang et al (2013) found that hepatic Socs3 expression was significantly lower in high fat diet induced obese rat than rat with control diet or high fat diet with exercise training.

Socs3 also have been showed relations with insulin signaling. In 3T3-L1 adipocyte, insulin increased Socs3 mRNA expression and translocated Socs3 from cytoplasm to the plasma membrane (Emanuelli et al. 2001). In addition, Socs3 is the well-known inhibitor of insulin signaling. On the molecular level, Socs3 impair insulin signaling by binding to insulin receptor substrates, such as IRS-1 and IRS-2. Therefore, IL-6, Tnf-α and leptin, which stimulated Socs3 expression, altogether could trigger insulin resistance. In current environment, many people, with high energy intake and low physical activity, suffered from expanded adipocyte and abnormal lipid metabolism. Therefore insulin resistance is deleterious since insulin resistance with surplus fat is intimately related to obesity and type 2 diabetes mellitus. Whereas in states of starvation or fasting, immune activation, and growth, insulin resistance is beneficial, because it could attribute to sparing glucose from blood and peripheral tissues, resulted in production of NADPH, nucleotides and oxaloacetate (Soeters et al. 2012, Tsatsoulis et al. 2013). Consequently, insulin resistance caused by Socs3 expression occasionally might play the beneficial role.

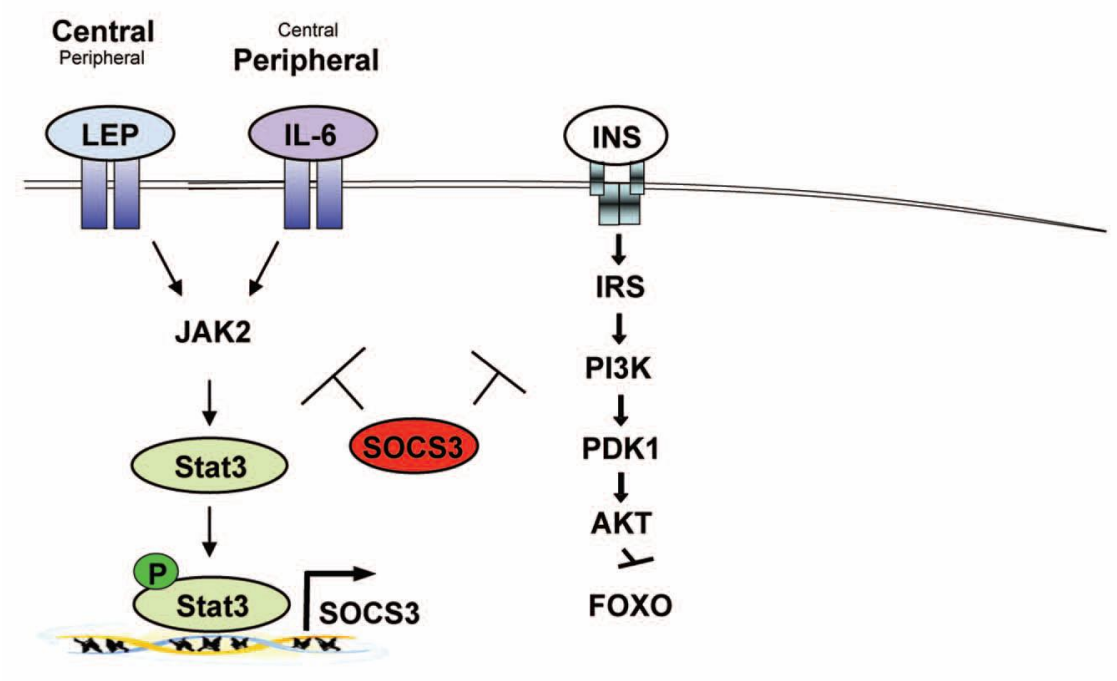


Figure 4. Chronic JAK-STAT3-SOCS3 signaling in obesity (Wunderlich et al. 2013)

3. Effects of calorie restriction on the lipid metabolism and inflammation

3-1. Calorie restriction

Calorie restriction (CR) is the reduction of 20~60% in total calories of ad libitum intake without malnutrition (Park et al. 2012). McCay et al. (1989) firstly found that CR increased maximum life span, offering rats with 20% less food than ad libitum controls ingested. Since then, many health benefits from CR are reported in flies, rodents, monkey, and humans (McCay et al. 1989, Spindler 2010). A diversity of protocols is adopted for CR in rodent model. First model of CR limits only nutrients that contribute to calorie production. Therefore during CR, micronutrients, such as minerals and vitamins, are needed to be supplemented with restricted formula diet of the America Institute of nutrition (AIN). Another CR model, also called as diet restriction (DR), restricted all kinds of nutrient without supplementation. Swiss mice fed 40% restriction of AIN-93M diets showed 20% of weight loss compared to control. But Swiss mice fed 40% restriction with supplementation of micronutrients were observed less than 10% of weight loss (Cerqueira et al. 2010). It means that large portion of diet restriction (more than 40%) in non-supplemented DR protocols could be nutritionally deficient.

3-2. Lipid metabolism and inflammation involved in calorie restriction

Calorie restriction (CR) has been known as effective manner of controlling energy intake followed by controlling lipid metabolism. CR induces weight loss and it decreases fat accumulation. Generally, accumulated adipose tissue promotes macrophage infiltration followed by secreting pro-inflammatory cytokines from macrophage in the adipocyte. Therefore CR reduced inflammation by suppressing expression of inflammatory cytokines in white adipose tissue (Huang et al. 2010, Park et al. 2012). However, it is controversial that whether reduced inflammation is resulted from CR itself or adipose mass loss induced by calorie restriction (Salas-Salvado et al. 2006). Some authors suggest that adipose mass loss through very low calorie diet, reduced levels of IL-6 and Tnf-α in adipose tissue of obese human(Bastard et al. 2000, Kern et al. 1995), whereas others have found an increase in Tnf-α expression(Bastard et al. 1999). Salas-Salvado et al.(2006) suggested that reduced inflammation seems to be largely due to CR rather than adipose mass loss, since CR in morbid obese non-diabetic patients resulted in reduced inflammation, which returned to the pre-treatment level shortly after stabilization of the body weight(Huang et al. 2010, Salas-Salvado et al. 2006).

A combination of CR and endurance exercise exhibits additive effects in prevention of obesity and insulin resistance with potent suppression of the

expression of inflammatory cytokines in WAT, but only had moderate to minimal effects in liver. These findings suggest that the adipose tissue is the main and common target of CR and endurance exercise. CR reduced both serum TG and cholesterol in mice on high fat diet to the level of mice on low fat diet. CR suppressed pro-inflammatory cytokines including osteopontin, Tnf-α, Mcp-1, IL-6, all of which are elevated in state of obesity and type 2 diabetes (Huang et al. 2010).

3-3. SIRT1 protein and calorie restriction

Many studies found that SIRT1 protein is deacetylated and activated by calorie restriction or fasting (Canto et al. 2010, Cohen et al. 2004). SIRT1 protein expression was higher in hepatic and adipose tissue of 40% of calorie restricted animals (Cohen et al. 2004). Canto et al found that low nutrient availability increased AMP/ATP ratio and it activates AMPK, enhancing lipid oxidation in the mitochondria and raising intracellular NAD+ level. Increased NAD+ level lead to SIRT1 activation resulted in deacetylated PGC-1a and FOXOs. SIRT1 is important for mediating the effects of CR because it could decrease the hepatic TG level (Schug et al. 2011). Ppara which contributes to fatty acid oxidation is activated by SIRT1 (Purushotham et al. 2009). Also Srebp-1c, which is the transcription factor of the genes required for lipid production, is inactivated by deacetylated SIRT1 (Horton et al. 1998, Ponugoti et al. 2010).

Ⅲ. Materials and methods

1. Animals

Male C57BL/6N mice (7 weeks old) were purchased from Central laboratory Inc. (Seoul, Korea) and were individually housed in cages in the specific pathogen free (SPF) animal facility at the college of veterinary medicine of Seoul National University. SPF room was controlled with constant temperature (25 ± 2℃), humidity (55 ± 10%), and 12 hour-dark/light cycle. After 1 week of acclimation with the control diet, mice were divided into 3 dietary group; Control diet group (Control group, n = 24), Calorie restriction group (CR group, n = 16), and high fat diet group (HFD group, n = 25) were fed with experimental diets for 16 weeks.

Food intake was measured every day and the body weight was recorded once a week. After 16 weeks on the experimental diets, mice were fasted for 12 hours and euthanized with CO_2 asphyxiation. All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University. (approval no. SNU-090710-1)

2. Diets

There were two types of experimental diets (Research Diets, Inc. New Brunswick, NJ, USA): control diet (10% calories from fat) and high fat diet (60% kcal from fat). The composition of experimental diets is shown in **Table 1**. Both diets were offered as solid pellet form and stored at 4℃.

Mice in HFD group were fed 60% fat diet *ad libitum* and those in the Control group were fed 10% fat diet *ad libitum*. Mice in Calorie restriction group were fed the same amount of diet as HFD group, but, with the control diet, which resulted in about 14.1% less caloric intake than the Control group and about 26.1% less caloric intake than HFD group. Calorie restriction was achieved by pairing each mouse in CR group with the mouse in HFD group, then providing the same amount of the food as the mouse in the HFD group ate the previous day to each mouse in CR group with the control. When fed *ad libitum*, generally mice on HFD eat less amount of food than those on the Control diet. Therefore, CR group were fed less amount of control diet than the Control group (14.1% less calorie intake).

Table 1. Composition of the experimental diets (g)[a]

	Normal diet		High-fat diet	
Component	gm%	Kcal%	gm%	Kcal%
Protein	19.2	20	26.2	20
Carbohydrate	67.3	70	26.3	20
Fat	4.3	10	34.9	60
Kcal/g diet	3.85		5.24	
Ingredient	Gm	Kcal		Kcal
Casein	200	800	200	800
L-Cysteine	3	12	3	12
Corn starch	315	1260	0	0
Maltodextrin	35	140	125	500
Sucrose	350	1400	68.8	275.2
Cellulose	50	0	50	0
Soybean oil	25	225	25	225
Lard	20	180	245	2205
Mineral mix[b]	10	0	10	0
Dicalcium phosphate	13	0	13	0
Calcium carbonate	5.5	0	5.5	0
Potassium citrate	16.5	0	16.5	0
Vitamin mix[c]	10	40	10	40
Choline bitartrate	2	0	2	0

a. Resource: Research Diets, Inc. New Brunswick, NJ, USA

b. mineral mix (10g) (Research diet, S10026) : sodium 1.0g, chloride 1.6g, magnesium 0.5g, sulfate 0.33, molybdate 1.6g, chromium 2.0g, copper 6.0g, iron 37mg, manganese 59mg, iodate 0.2mg, fluoride 0.9mg, selenite 0.16mg, zinc 29mg, sucrose 3.99g.

c. vitamin mix (10g) (Research diet, V10001) : vitamin A 4000IU, Vitamin D3 1000IU, Vitamin E 50IU, Menadione 0.5mg, Biotin .2mg, Vitamin B12 10ug, Folate 2mg, Niacin 30mg, Panthothenic acid 16mg, Vitamin B6 7mg, Vitamin B12 6mg, Vitamin B1 6mg, sucrose 9.78g

3. Methods

3-1. Tissue collection

After mice were euthanized by asphyxiation with CO_2, blood was collected via cardiac puncture. Serum was separated by centrifugation at 2000 rpm for 20 minutes after coagulation at room temperature for 2 hours. Serum was stored at -80℃. Liver was weighed and dissected into four lobes. Epididymal, subcutaneous, retroperitoneal, and brown fat pads were collected and weighted. All the samples are stored at -80℃.

3-2. Serum analyses

3-2-1. Determination of serum Triglyceride, Cholesterol and Free fatty acids

Serum TG concentration was determined using commercial kit (Asan pharmaceutical, Korea) based on enzymatic assay. Two μL of serum or standards was added to each well of 96-well plate and 300 μL of enzyme solution was added to each well, then incubated for 10 minutes at 37 ℃. The absorbance was measured at 550 nm (spectramax 190; molecular devices, CA, USA). Concentration of TG was calculated using standard curve.

Serum Cholesterol concentration was determined using commercial kit (Asan pharmaceutical, Korea) based on enzymatic assay. Two μL of serum or standards was added to each well of 96-well plate and 300 μL of enzyme

solution was added to each well, then incubated for 5 minutes at 37 ℃. The absorbance was measured at 500 nm (spectramax 190; molecular devices, CA, USA). Concentration of Cholesterol was calculated using standard curve.

Serum free fatty-acids concentration was determined by SICDIA NEFAZYME kit (Shin Yang Chemical, Busan). Four μL of serum or standards was added to each well of 96-well plate and 200 μL of NEFA enzyme solution1 and NEFA enzyme solution2 were added to each well, then incubated for 10 minutes at 38 ℃. The absorbance was measured at 546 nm (spectramax 190; molecular devices, CA, USA) and concentration of serum was calculated using standard curve.

3-2-2. Determination of serum Glucose, AST and ALT

Serum glucose concentration was determined using commercial kit (Asan pharmaceutical, Korea) based on enzymatic assay. Two μL of serum or standards was added to each well of 96-well plate and 300 μL of enzyme solution was added to each well, then incubated for 10 minutes at 37 ℃. The absorbance was measured at 500 nm (spectramax 190; molecular devices, CA, USA). Concentration of glucose was calculated using standard curve.

Serum AST, ALT concentrations were determined using commercial kit (Asan pharmaceutical, Korea) based on enzymatic assay. Thirty μL of

substrate solution were incubated for 5 minutes at 37 ℃. Six μL of serum was added to each well of 96-well plate and incubated for 60 minutes (or 30 minutes) at 37 ℃. Thirty μL of enzyme solution was added and incubated for 20 minutes at room temperature, then 300 μL of NaOH solution was added. After incubation for 10 minutes, the absorbance was measured at 505 nm (spectramax 190; molecular devices, CA, USA). Concentration of AST and ALT were calculated using standard curve.

3-3. Serum adipokines and hepatokine analyses

3-3-1. Determination of serum leptin and adiponectin

Serum leptin concentration was determined by Quantikine® mouse leptin kit (R&D Systems, MN, USA). In detail, 50 μL of diluted solution and 50 μL of 20-fold diluted serum or standard solution were added to 96-well plate coated with mice leptin specific antibody. After incubation of 2 hours at room temperature, each well of 96-well plate was washed 5 times by filling each well with washing buffer. 100 μL of mice leptin specific antibody attached with HRP (horseradish peroxidase) was added to each well and incubated for 2 hours at room temperature. After incubation, each well of 96-well plate was washed 5 times again with washing buffer and 100 μL of substrate solution of HRP was added, then incubated for 30 minutes at room temperature. Afterward 100 μL of diluted hydrochloric acid solution was

added to stop the reaction. The absorbance was measured at 450nm and 570nm using a microplate reader (spectramax 190; molecular devices, CA, USA). Leptin concentration was calculated using standard curve.

Serum adiponectin concentration was determined by Quantikine® mouse adiponectin kit (R&D Systems, MN, USA). In detail, 50 μL of diluted solution and 50 μL of 2000-fold serum or standard solution were added to 96-well plate coated with mice adiponectin specific antibody. After incubation of 3 hours at room temperature, each well of 96-well plate was washed five times by filling each well with each buffer. 100 μL of mice adiponectin specific antibody attached with HRP (horseradish peroxidase) was added to each well and incubated for 1 hour. Each well of 96-well plate was washed 5 times again and 100 μL of substrate solution of HRP was added. Afterward incubation for 30 minutes, 100 μL of diluted hydrochloric acid solution was added to stop the reaction. The absorbance was measured at 450nm and 570nm using a microplate reader (spectramax 190; molecular devices, CA, USA). Adiponectin concentration was calculated using standard curve.

3-3-2. Determination of serum fetuin-A concentration

Serum fetuin-A concentration was determined by mouse fetuin-A/AHSG Duoset (R&D systems). In detail, 100 μL of mice fetuin-A specific antibody was added to 96-well plate and incubated for 16 hours at room temperature.

Each well was aspirated and washed with wash buffer for 3 times, then 300 μL of reagent diluent was added. After incubation for 1 hour, each well was aspirated and washed for 3 times again. One hundred μL of 5000-fold serum or standard solution was added and incubated for 2 hours at room temperature. After 3 times of washes with wash buffer, 100 μL of mice fetuin-A specific antibody attached with HRP (horseradish peroxidase) was added to each well and incubated for 2 hours. Each well of 96-well plate was washed 3 times again with wash buffer and 100 μL of substrate solution of HRP was added. After incubation for 20 minutes, 50 μL of diluted sulfuric acid solution was added to stop the reaction. The absorbance was measured at 450nm and 570 nm using a microplate reader (spectramax 190; molecular devices, CA, USA). Fetuin-A concentration was calculated using standard curve.

3-4. Hepatic lipid analyses

Total hepatic lipids were extracted according to the method of Folch et al. (Folch et al. 1957). Thirty mg of liver tissue was homogenized in 60 μL of PBS. Eight hundreds μL of chloroform and 400 μL of methanol were added to homogenized sample and incubated for 17 hours at room temperature. After centrifugation at 2,000 × g for 10 minutes at 4℃, bottom layer (lower transparent part) was transferred into fresh tubes and evaporated with

nitrogen gas. One hundred μL of isopropanol was added to the lipid pellets and amounts of TG and cholesterol from lipids were determined by enzymatic colorimetric method (same as the method of 3-2).

3-5. Total RNA extraction and real time PCR

3-5-1. Extraction of total RNA

Total RNA from liver and epididymal fat tissue was extracted with TRIZOL (Invitrogen, CA, USA). Fifty mg of liver tissue or 20 mg of epididymal fat tissue was homogenized in 1mL of TRIZOL reagent and incubated for 5 minutes at room temperature. In the case of epididymal fat tissue, homogenized sample was centrifuged at 12,000 × g for 10 minutes at 4℃. Two hundreds μL of chloroform was added and incubated for 3 minutes at room temperature. After centrifugation at 12,000 × g for 15 minutes at 4℃, aqueous phase (upper transparent class) was transferred into fresh tubes and 500 μL of isopropanol was added. After 10 minutes of incubation, samples were centrifuged at 12,000 × g for 10 minutes at 4℃ and the supernatant was removed. One mL of 75% ethanol was added to wash the pellet. Samples were centrifuged at 7,500 × g for 2 minutes at 4℃ and supernatant was removed. RNA pellet was dried for 15 minute and dissolved in 20 μL of diethylpyrocarbonate (DEPC)-treated water. The absorbance of the diluted RNA solution was measured at 260nm to calculate

the concentration of the RNA.

3-5-2. cDNA synthesis

cDNA was synthesized using PrimeScript™ Ⅱ 1st strand cDNA synthesis kit (TAKARA bio Inc., Japan). Oligo dT primer (50 μM) 1 μL, dNTP mixture (10 μM) 1 uL and RNase-free dH_2O 3 μL were added to 2 μg of RNA (5 μg/μL). And the mixed solution was incubated for 5 minutes at 65 ℃ and for 5 minutes at -20 ℃. PrimeScript™ Ⅱ buffer 4 μL, RNase inhibitor (40 U/ μL) 0.5 μL, PrimeScript™ Ⅱ RTase (200 U/ μL) 1 μL and RNase-free dH20 4.5 μL were added to incubated solution and synthesized cDNA using Applied Biosystems thermal cycler 2720 (Life technology co., CA, USA). The condition for reverse transcription was 42℃ for 50 minutes, 95℃ for 5 minutes, and 4℃ for 30 minutes.

3-5-3. Real time PCR

To identify the effects of calorie restriction on lipid metabolism and inflammation, the mRNA levels of *Pparg, Ppara, Cpt1a, Srebf-1c, Ahsg, Adipor2, Fasn, Fabp1* in liver and *Mcp-1, IL-6, IL-1β, Socs3, Tnf-α,* and *Lepr* in liver and epididymal fat were determined by real-time PCR with a SYBR Premix Ex Taq (Takara, Japan) and StepOne Real-time PCR System (Applied Biosystems, CA, USA).

In detail, all reactions were performed in total of 20 μL reaction volume

containing 1 μL of diluted cDNA, 10 μL of SYBR Premix Ex Taq, 0.4 μL of 10μM forward primer, 0.4 μL of 10μM reverse primer, 0.4 μL of ROX reference dye, and 7.8 μL of autoclaved distilled water. Conditions for the PCR reaction were 95℃ for 30 seconds to initiation and 95℃ for 10 seconds to denaturation, 60℃ for 60 seconds to annealing and 95 ℃ for 15 seconds to elongation up to 40 cycles.

To normalize the results, Δthreshold cycle (Ct) was calculated by subtracting the Ct value of house-keeping gene *Gapdh* from that of specific gene. The relative expression of each gene was calculated from $2^{-\Delta\Delta CT}$. The specific primer sequences are shown in Table 2.

GENE	Function	Forward primer	Reverse primer
Pparg	De novo lipogenesis	CAGCAGGTTGTCTTGGATGTC	AGCCCTTTGGTGACTTTATGG
Pparα	Fatty acid oxidation	GCAGTGGAAGAATCGGACCT	CAACCCGCCTTTTGTCATAC
AdipoR2	Adiponectin receptor	CTGTGTGCTGGGCATTGCAG	AGCCTATCTGCCCTATGGTG
Fabp1	Fatty acid transport	GAACTCATTGCGGACCACTT	CATCCAGAAAGGGAAGGACAT
Fasn	De novo lipogenesis	GCGGTGTGAAAACGAACTTT	CTGTCTGGGCATAACGGTCT
Cpt1a	Fatty acid oxidation	GATGTTCTTCGTCTGGCTTGA	CTTATCGTGGTGGTGGGTGT
Ahsg	Fatty liver indicator	TTGCTCAGCTCTGGGGCT	GGCAAGTGGTCTCCAGTGTG
Srebf-1c	De novo lipogenesis	GTCTCCACCACTTCGGGTTT	CGACTACATCCGCTTCTTGC
Mcp-1	Pro-inflammatory chemokine	AGGCATCACAGTCCGAGTCAC	CCTTTTCCACAACCACCTCAAG
Socs3	Suppressor of cytokine	CGGACCTACTGACCGAGAGA	CACACAAGGAGCCAAACACA
IL-1β	Pro-inflammatory cytokine	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	Pro-inflammatory cytokine	CATTTCCACGATTTCCCAGAGA	TCCATCCAGTTGCCTTCTTGGG
Tnf-α	Pro-inflammatory cytokine	CTGGAAAGGTCTGAAGGTAGGAAGG	AACACAAGATGCTGGGACAGTGA
Lepr	Leptin receptor	ATTCCCAAAGCAACAGTGGA	ACCGAACACAACCGATGACT
Gapdh	Endogenous control	GGAGAAACCTGCCAAGTA	AAGAGTGGGAGTTGCTGTTG

Table 2. Primer sequences used in real time PCR[1]

[1]*Pparg*, peroxisome proliferator-activated receptor gamma; *Pparα*, peroxisome proliferator-activated receptor alpha; *AdipoR2*, adiponectin receptor2; *Fabp1*, fatty acid binding protein; *Fasn*, fatty acid synthase; *Cpt-1a*, carnitine palmitoyltransferase 1; *Ahsg*, alpha-2-HS-glycoprotein; *Srebf-1c*, sterol regulatory element-binding protein; *Mcp-1*, monocyte chemoattractant protein 1; *Socs3*, suppressor of cytokine signaling 3; *IL-1β,* interleukin 1beta; *IL-6*, interleukin 6; *Lepr*, *Tnf-α*, Tumor necrosis factor; *Lepr*, leptin receptor; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.

3-6. Total Protein extraction and immunoblotting

3-6-1. Protein extraction

Fifty mg of liver tissue (50mg) was homogenized with 500 μL RIPA buffer for 15 seconds, 5 times on ice. Homogenized liver tissue was centrifuged at 16,000 x g for 5 minutes and supernatant was collected into fresh tube. Ten μL of protein sample or several dilution of BSA standard solutions (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1mg/mL) and 200 μL of dye reagent were added to 96-well plate. Afterward incubation for 5 minutes, the absorbance was measured at 595nm using a microplate reader (spectramax 190; molecular devices, CA, USA). Total protein concentration was calculated using standard curve. .

3-6-2. Western blot

Western blot analysis was conducted to determine protein expression levels of SIRT1, AMPK, pSTAT3/STAT3.

Ten μL of protein (40 μg/ 20 μL) was mixed with 10 μL of sample buffer containing 5% of β-mercaptoethanol and denatured for five minute at 100 ℃. Twenty micrograms of protein was separated by electrophoresis on a 10% SDS-polyacrylamide gel. The condition of electrophoresis was 50 V for 35 minutes and 120 V for 85 minutes. After electrophoresis, proteins on the gel were transferred to PVDF membrane in the buffer containing 100 mL of methanol, 100 mL of running buffer (10X) and 800 mL of distilled water at

the condition of 90 V for 2 hours. Transferred protein in PVDF membrane was identified by Ponceau S solution. After 3 times of washes with washing buffer, the membrane was blocked with 5% skim milk (or 5% BSA) in Tris buffered saline solution containing 0.1% Tween-20, pH 7.6 (TBST) for at least 1 hour. Next, membrane was incubated with rabbit anti-SIRT1 (millipore, USA, 1:1000) or rabbit anti-STAT3 (Cell signaling technology, MA, USA, 1:1000) or rabbit anti-pSTAT3 (Cell signaling technology, MA, USA, 1:2000) in TBST containing 5% skim milk (or 5% BSA) for overnight at 4℃. After 3 times of washes with washing buffer, membrane was incubated with HRP-conjugated Anti rabbit IgG (Cell signaling technology, MA, USA, 1:3000) in TBST containing 5% skim milk(or 5% BSA) for 1 hour at room temperature. Specific bands on the membrane were visualized with an enhanced chemiluminescence (ECL) solution (Santa Cruz, CA, USA).

4. Statistical analysis

All data were analyzed using SPSS 19.0 program (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) was used to evaluate the overall difference among groups, followed by Fisher's LSD multiple test to compare among individual groups. A Pearson correlation was used to determine the association between parameters. The results from all the comparisons were considered significant at $P < 0.05$. Data were reported as the mean ± SE.

Ⅳ. Results

1. Body weight changes, food intake, white adipose tissue weight, and liver weight

There was no difference in the average weight among the groups at the beginning of the study **(Figure 5)**. After 16 weeks of feeding, HFD group had significantly higher weight gain ($P < 0.001$), more white adipose tissue ($P < 0.001$), brown adipose tissue ($P < 0.001$), and higher liver weight ($P < 0.001$) than Control and CR groups **(Table 3).** Mice in CR group had significantly lower weight gain ($P < 0.001$), less white adipose tissue ($P < 0.001$), less brown adipose tissue ($P < 0.001$), and lower liver weight ($P < 0.001$) than those in Control group **(Table 3)**.

Average daily diet intake of the Control group was significantly higher than that of the HFD group. Average daily food intake was same in CR and HFD groups due to the pair-feeding design. CR group had significantly lower daily energy intake than the Control (14.1% less, $P < 0.001$) and HFD groups (26.1% less, $P < 0.001$).

There was no significant difference in liver weight per body weight (g/100g body weight) between HFD and Control groups, whereas CR group had significantly lower liver weight per body weight than both HFD ($P < 0.001$) and Control groups ($P < 0.01$).

Table 3. Body weight, weight gain, liver weight, liver weight per body weight, diet intake, and adipose tissue weight of mice in CR, Control, and HFD groups[1]

	Calorie restriction (n =16)	Control (n = 24)	High fat diet (n = 25)	P value
Body weight at 0 week (g)	22.68 ± 0.20	22.40 ± 0.22	22.26 ± 0.21	0.44
Body weight at 16 week (g)	27.53 ± 0.76^{a}	37.08 ± 0.56^{b}	48.80 ± 0.49^{c}	< 0.001
Body weight gain (g)	4.8 ± 0.8^{a}	14.7 ± 0.5^{b}	26.5 ± 0.5^{c}	< 0.001
Liver weight (g)	1.15 ± 0.05^{a}	1.91 ± 0.06^{b}	2.73 ± 0.12^{c}	< 0.001
Liver weight per body weight (g/100g body weight)	4.18 ± 0.15^{a}	5.11 ± 0.11^{b}	5.58 ± 0.23^{b}	< 0.001
Average daily diet intake (g/day)	2.98 ± 0.04^{a}	3.50 ± 0.04^{b}	2.98± 0.03^{a}	< 0.001
Daily energy intake (kcal/day)	11.43 ± 0.16^{a}	13.49 ± 0.14^{b}	15.59 ± 0.15^{c}	< 0.001
White adipose tissue weight[2] (g)	1.76 ± 0.14^{a}	3.94 ± 0.15^{b}	6.87 ± 0.11^{c}	< 0.001
Brown adipose tissue weight (g)	0.18 ± 0.01^{a}	0.34 ± 0.02^{b}	0.39 ± 0.01^{c}	< 0.001

Data are presented as mean ± SE.
[1]One-way ANOVA was used to determine the significant effect of group.
[2]White adipose tissue included epididymal fat, subcutaneous fat, retroperitoneum fat, and perinephric fat.
Different letters indicate significant differences at $P < 0.05$.

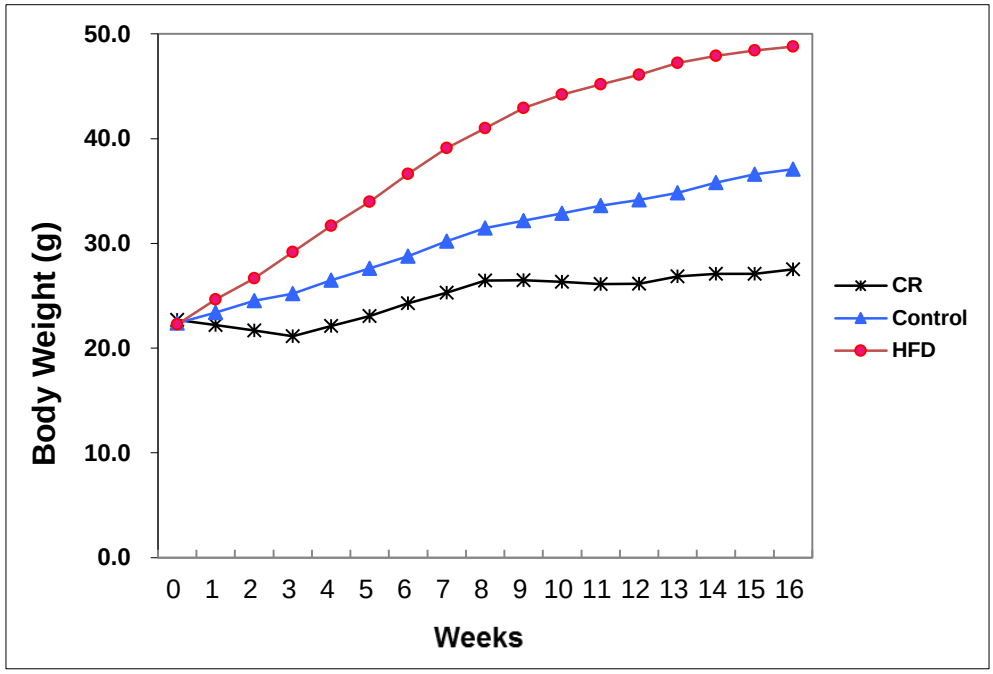


Figure 5. Weekly body weight
Body weight of Control group (n = 24), HFD group (n = 25), and CR group (n = 16) during 16 weeks of experimental period.

2. Serum lipid analyses

Serum TG, cholesterol, and non-esterified fatty acid (NEFA) levels were significantly different among groups. CR group and Control group had the lower levels of serum TG than HFD group ($P < 0.05$). And CR group had lower level of serum cholesterol than Control and HFD groups ($P < 0.001$), while there was no difference between HFD group and Control group. However serum NEFA concentration was significantly higher in CR group compared with other two groups ($P < 0.01$) **(Figure 6)**.

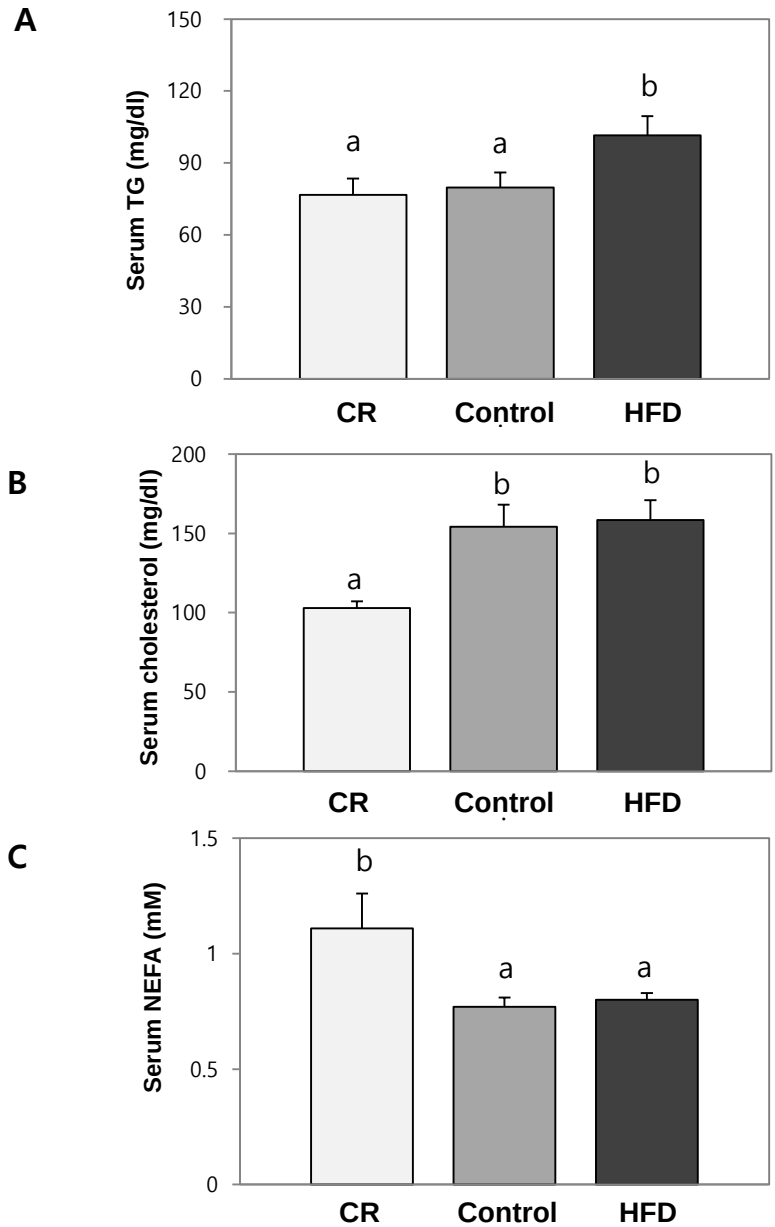


Figure 6. Serum lipid levels. (A) Serum TG (mg/dl), (B) Serum cholesterol (mg/dl), and (C) Serum NEFA (mM)

Data are presented as mean ± SE, n = 7 for each group. One-way ANOVA was used to determine the significant difference among groups. Different letters indicate significant difference at $P < 0.05$ by Fisher's LSD multiple comparison test.

3. Serum glucose, AST, and ALT concentrations

Serum glucose levels were significantly different among groups ($P < 0.001$) **(Figure 7)**. Serum glucose level was significantly lower in CR group than Control and HFD groups ($P < 0.001$). Serum glucose level showed positive correlations with white adipose tissue weight ($r = 0.746$, $P < 0.001$), serum TG level ($r = 0.486$, $P < 0.01$), and serum cholesterol level ($r = 0.436$, $P < 0.05$).

Serum alanine transaminase (ALT) levels were significantly different among groups ($P < 0.01$), but serum aspartate transaminase (AST) levels were not different among groups. Serum ALT level was significantly lower in CR group ($P < 0.001$) and Control group ($P < 0.01$) than HFD group. Also serum ALT level showed positive correlations with serum glucose level ($r = 0.563$, $P < 0.01$), white adipose tissue weight ($r = 0.695$, $P < 0.001$), serum TG level ($r = 0.534$, $P < 0.05$).

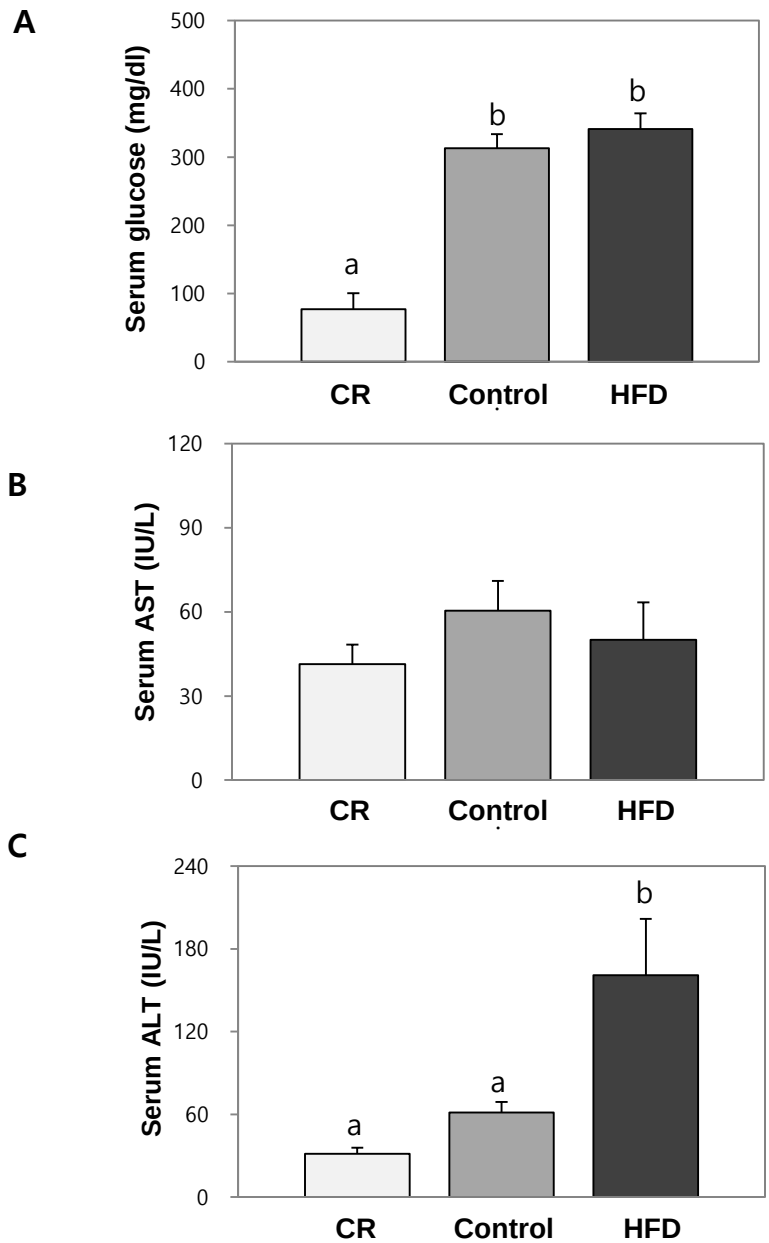


Figure 7. Serum glucose, aspartate transaminase (AST), and alanine transaminase (ALT) levels. (A) Serum glucose (mg/dl), (B) Serum AST (IU/L), and (C) Serum ALT (IU/L)

Data are presented as mean ± SE, n = 7 for each group. One-way ANOVA was used to determine the significant difference among groups. Different letters indicate significant difference at $P < 0.05$ by Fisher's LSD multiple comparison test.

Table 4. Serum TG, cholesterol, NEFA , glucose, AST, and ALT concentrations[1]

	Calorie restriction	Control	High fat diet	P value
Serum TG (mg/dl)	76.74 ± 6.79[a]	79.74 ± 6.27[a]	101.46 ± 8.11[b]	0.048
Serum cholesterol (mg/dl)	102.84 ± 4.35[a]	154.19 ± 13.96[b]	158.48 ± 12.37[b]	0.004
Serum NEFA (mM)	1.09 ± 0.12[b]	0.74 ± 0.05[a]	0.77 ± 0.06[a]	0.011
Serum glucose (mg/dl)	140.57 ± 24.38[b]	313.07 ± 18.81[a]	340.86 ± 21.71[a]	< 0.001
Serum AST (IU/L)	41.30 ± 6.45	60.46 ± 9.76	50.07 ± 12.30	0.403
Serum ALT (IU/L)	31.45 ± 10.60[a]	61.22 ± 7.25[a]	166.65± 36.32[b]	0.001

Data are presented as mean ± SE, n=7 for each group.
[1]One-way ANOVA was used to determine the significant effect of group.
Different letters indicate significant differences at $P < 0.05$

4. Serum leptin and adiponectin concentrations

Serum leptin levels were significantly different among groups ($P < 0.001$). HFD group had the highest level and CR group had the lowest level. The average serum leptin level of HFD group was 82.8% higher than that of Control group. And the average level of leptin in CR group was 72% lower than Control group **(Table 5)**. A significantly positive correlation was observed between the amount of white adipose tissue and serum leptin level ($r = 0.979$, $P < 0.001$) **(Figure 8).** Also serum leptin level had positive correlations with serum TG, cholesterol and glucose levels ($r = 0.556$, $P < 0.01$; $r = 0.592$, $P < 0.01$; $r = 0.783$, $P < 0.001$, respectively).

Serum adiponectin concentration was lower in HFD group than control group ($P < 0.001$) and CR group ($P < 0.001$). Also CR group had higher serum adiponectin level than Control group (45% higher, $P < 0.01$) **(Table 5)**. There was a significantly negative correlation between serum adiponectin level and the amount of white adipose tissue ($r = -0.666$, $P < 0.01$) **(Figure 8).**

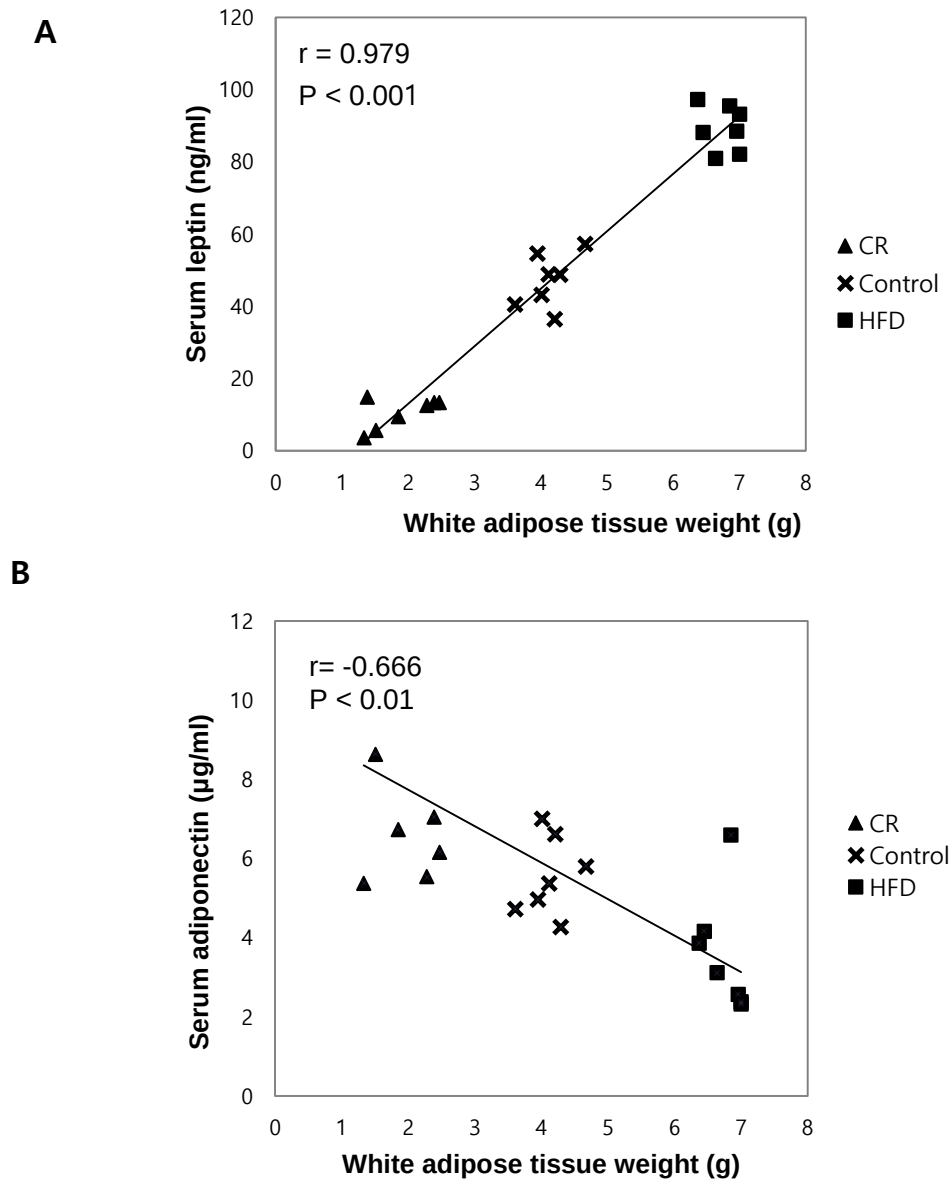


Figure 8. Correlations between adipokines and white adipose tissue weight. (A) Correlation between leptin level and white adipose tissue amounts(g) (B) Correlation between adiponectin level and white adipose tissue amounts(g)
Pearson correlation coefficient, r, and P-value are indicated for each region (n = 21).

Table 5. Serum leptin, adiponectin, and fetuin-A concentrations[1]

	Calorie restriction (n = 16)	Control (n = 24)	High fat diet (n = 25)	P value
Serum leptin (ng/ ml)	12.86 ± 2.53[a]	45.43 ± 2.84[b]	83.05 ± 2.12[c]	< 0.001
Serum adiponectin (μg/ ml)	8.27 ± 1.12[c]	5.72 ± 0.35[b]	3.67 ± 0.24[a]	< 0.001
Serum fetuin-A (μg/ ml)	125.10 ± 3.89[a]	144.80 ± 3.76[b]	154.85 ± 6.47[b]	0.001

Data are presented as mean ± SE.
[1]One-way ANOVA was used to determine the significant effect of group.
Different letters indicate significant differences at $P < 0.05$

5. Serum fetuin-A concentration and hepatic mRNA expression of fetuin-A

Serum fetuin-A level, which was known to be elevated in non-alcoholic fatty liver disease (NAFLD), was lower in CR group than both control ($P < 0.05$) and HFD groups ($P < 0.001$). There was no difference in serum fetuin-A concentration between HFD and Control groups **(Table 5)**. Serum fetuin-A level was positively correlated with body weight ($r = 0.692$, $P<0.01$), white adipose tissue weight ($r = 0.625$, $P < 0.01$), brown adipose tissue ($r = 0.644$, $P < 0.01$), liver weight per body weight ($r = 0.746$, $P < 0.001$), and serum leptin level ($r = -0.584$, $P < 0.01$), whereas negatively correlated with serum adiponectin level ($r = -0.452$, $P < 0.05$).

Hepatic *Ahsg* (Fetuin-A, alpha-2-HS-glycoprotein) mRNA expression was not affected by diet type or total calorie intake and didn't have correlation with other values **(Figure 9).**

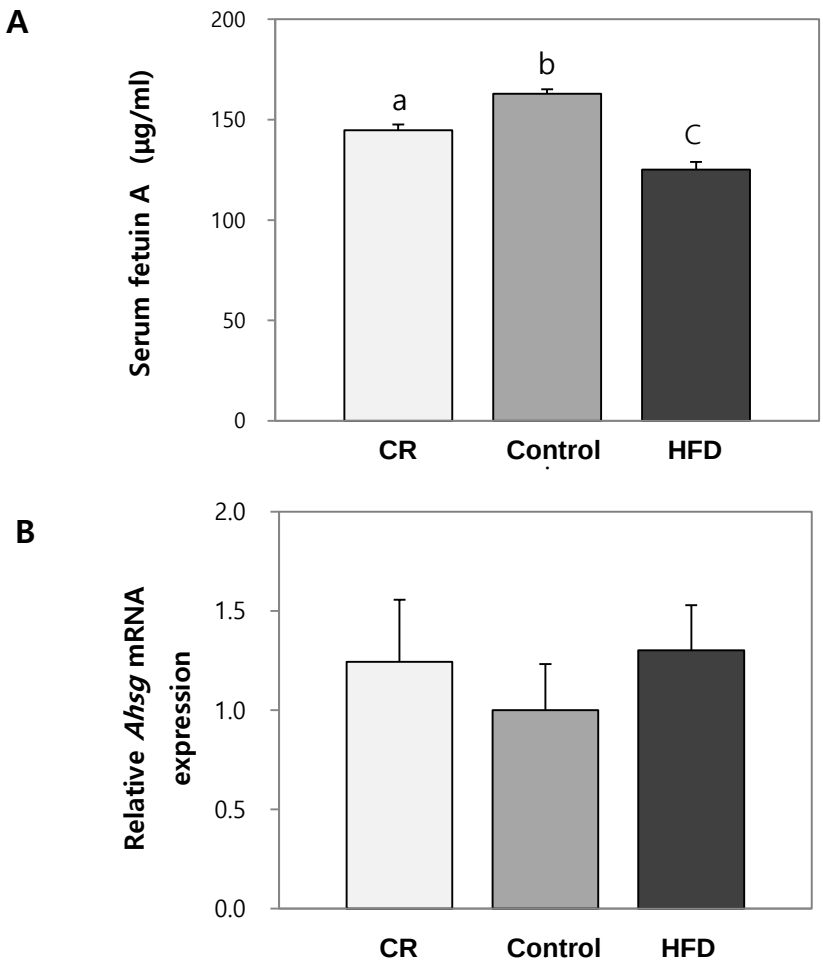


Figure 9. Serum fetuin-A level and hepatic mRNA expression of Ahsg. (A) Serum fetuin-A level, and (B) Relative fetuin-A expression.

Data are presented as mean ± SE, n = 7 for each group. One-way ANOVA was used to determine the significant difference among groups. Different letters indicate significant difference at $P < 0.05$ by Fisher's LSD multiple comparison test.

6. Hepatic lipid analyses

Hepatic TG and cholesterol concentrations were significantly different among groups ($P < 0.001$, and $P < 0.01$, respectively) **(Figure 10)**. In CR group, liver TG level (mg/ g tissue) and liver cholesterol level (mg/ g tissue) were lower than Control and HFD groups **(Table 6).**

A significantly positive correlation was observed between liver TG and liver cholesterol levels ($r = 0.863$, $P < 0.001$). Liver TG level (mg/ g tissue) showed significantly positive correlation with serum ALT ($r = 0.476$, $P < 0.05$) and fetuin-A levels ($r = 0.434$, $P < 0.05$). In addition, liver cholesterol level (mg/ g tissue) also had positive correlation with serum ALT ($r = 0.540$, $P < 0.05$) and fetuin-A levels ($r = 0.483$, $P < 0.05$).

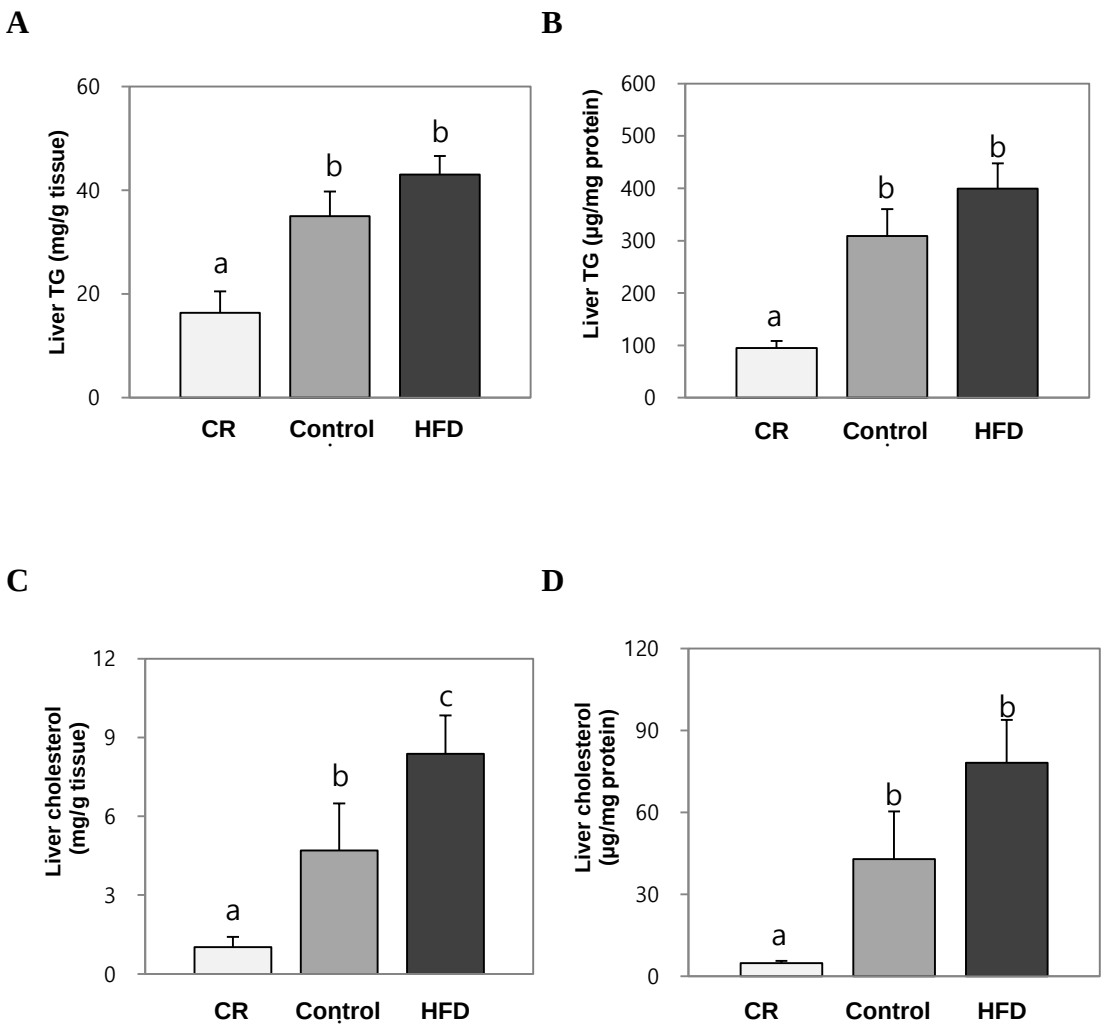


Figure 10. Hepatic lipid levels. (A) Serum TG level (mg/g tissue), (B) Serum TG level (µg/mg protein), (C) Serum cholesterol level (mg/g tissue), and (D) Serum cholesterol level (µg/mg protein) Data are presented as mean ± SE, n = 6-7 for each group. One-way ANOVA was used to determine the significant difference among groups. Different letters indicate significant difference at $P < 0.05$ by Fisher's LSD multiple comparison test.

Table 6. Liver triglyceride and cholesterol concentrations[1]

	Calorie restriction	Control	High fat diet	P value
Liver triglyceride (mg/tissue)	16.37 ± 3.83[a]	35.02 ± 4.37[b]	43.04 ± 3.33[b]	< 0.001
Liver triglyceride (μg/mg protein)	94.66 ± 12.41[a]	309.20 ± 47.02[b]	399.62 ± 44.39[b]	< 0.001
Liver cholesterol (mg/tissue)	1.02 ± 0.36[a]	4.701 ± 1.66[b]	8.37 ± 1.24[c]	0.004
Liver cholesterol (μg/mg protein)	4.73 ± 0.74[a]	42.91 ± 16.14[b]	78.15 ± 14.60[b]	0.005

Data are presented as mean ± SE, n = 6~7 for each group.
[1]One-way ANOVA was used to determine the significant effect of group.
Different letters indicate significant differences at $P < 0.05$

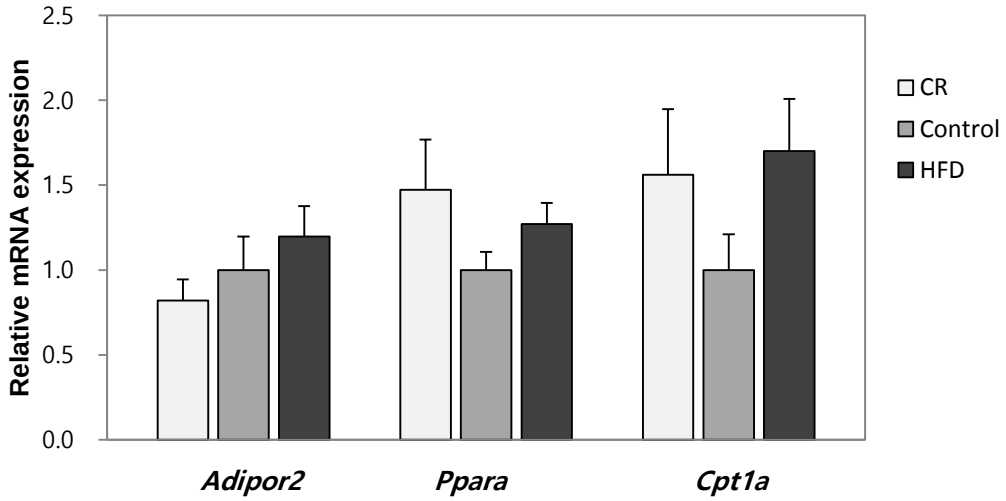
7. Expression of genes involved in hepatic lipid metabolism

Hepatic mRNA levels of *Adipor2, Ppara*, and *Cpt1a* were determined to look at the influence of obesity and calorie restriction on lipid oxidation. The mRNA level of *Adipor2*, the adiponectin receptor 2, was not significantly different among groups. The mRNA levels of *Adipor2* negatively correlated with adiponectin ($r = -0.459$, $P < 0.05$). Expressions of *Ppara*, which promotes TG degradation and hepatic fatty acid oxidation, and *Cpt1a*, which contributes to beta-oxidation of long chain fatty acid, were not affected by diet type and calorie restriction **(Figure 11).** *Ppara* mRNA level showed a significantly positive correlation with *Cpt1a* mRNA level ($r = 0.822$, $P < 0.001$). There was no correlation between serum NEFA level and *Ppara* mRNA level although *Ppara* is known to degrade TG and contribute to serum fatty acid concentration (Bragt et al. 2008).

In order to investigate whether HFD-induced obesity and calorie restriction affected lipid synthesis, the mRNA levels of *Pparg*, *Srebf-1c*, *Fasn*, and *Fabp1* were determined. The mRNA level of *Pparg*, known to be involved in lipid synthesis in many tissues, was higher in HFD group than Control group ($P < 0.05$) and CR group ($P < 0.001$). Whereas *Pparg* mRNA level of Control group was not significantly higher than that of CR group. *Pparg* mRNA level correlated positively with body weight ($r = 0.754$, $P < 0.001$), white adipose tissue amount ($r = 0.754$, $P < 0.001$), serum leptin level ($r =$

0.740, P < 0.001), serum ALT level (r = 0.612, P < 0.01), liver TG (r = 0.524, P < 0.05) and cholesterol levels (r = 0.428, P < 0.05) and hepatic *Adipor2* mRNA level (r = 0.556, P < 0.01) and negatively correlated with serum adiponectin level (r = -0.591, P < 0.01). The mRNA level of *Srebf-1c*, which is responsible for activating lipid synthesis pathway and storing lipid in liver, was also higher in HFD group than control group and CR group (P < 0.05, P < 0.05). There was no difference in *Srebf-1c* mRNA levels between control group and CR group. The mRNA levels of *Fasn* and *Fabp1* were not affected by HFD-induced obesity and calorie restriction **(Figure 11).** *Fasn* mRNA level showed positive correlation with *Srebf-1c* mRNA level (r = 0.444, P < 0.05) and *Fabp1* mRNA level showed positive correlation with *Ppara* mRNA level (r = 0.451, P < 0.05).

A



B

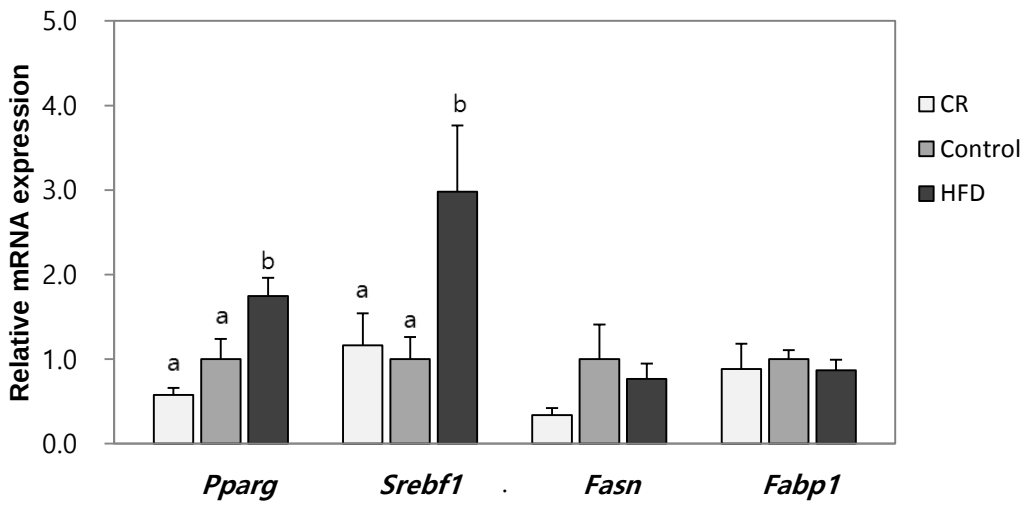


Figure 11. Hepatic mRNA levels of genes involved in lipid metabolism. **(A) Relative mRNA expressions of genes involved in fatty acid oxidation, and (B) Relative mRNA expressions of genes involved in lipogenic pathway.** Data are presented as mean ± SE, n = 7 for each group. One-way ANOVA was used to determine the significant difference among groups. Different letters indicate significant difference at $P < 0.05$ by Fisher's LSD multiple comparison test. All values were normalized to the levels of house-keeping gene *Gapdh* and expressed as relative mRNA level compared to the average level of Control group. *AdipoR2,*

adiponectin receptor2; *Ppara*, peroxisome proliferator-activated receptor alpha; *Cpt1a*, carnitine palmitoyltransferase 1; *Pparg*, peroxisome proliferator-activated receptor gamma; *Srebf-1c*, sterol regulator element-binding protein; *Fasn*, fatty acid synthase; *Fabp1*, fatty acid binding protein.

8. Expression of SIRT1 protein related to calorie restriction in liver

The protein level of SIRT1, known as a regulator of hepatic lipid metabolism, was determined by western blot. Expression of SIRT1 was not different among group (P = 0.200) **(Figure 12).**

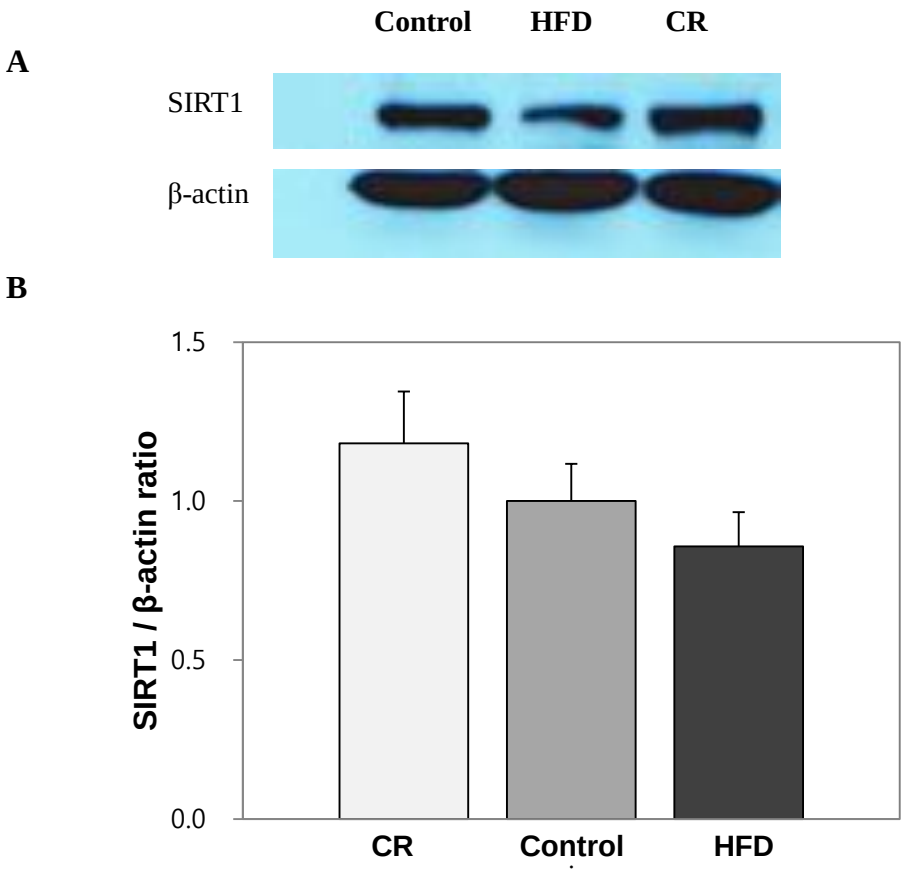


Figure 12. The protein expression levels of SIRT1 in liver. (A) Western blotting for SIRT1 and β-actin, and (B) Densitometric analysis of SIRT1 protein expression

The intensity of SIRT was densitometrically measured and normalized to the protein expression level of β-actin. Values are mean ± SE, n = 7 for each group. One-way ANOVA was used to determine the significant difference among groups. Different letters indicate significant difference at $P < 0.05$ by Fisher's LSD multiple comparison test.

9. Expression of genes involved in inflammation

To confirm whether calorie restriction attenuated inflammation, we determined mRNA levels of *Mcp-1*, inflammatory cytokines *IL-6, IL-1β*, and *Tnf-α* and suppressor of cytokine signaling *(Socs)3* molecule in liver and epididymal adipose tissue **(Figure 13).**

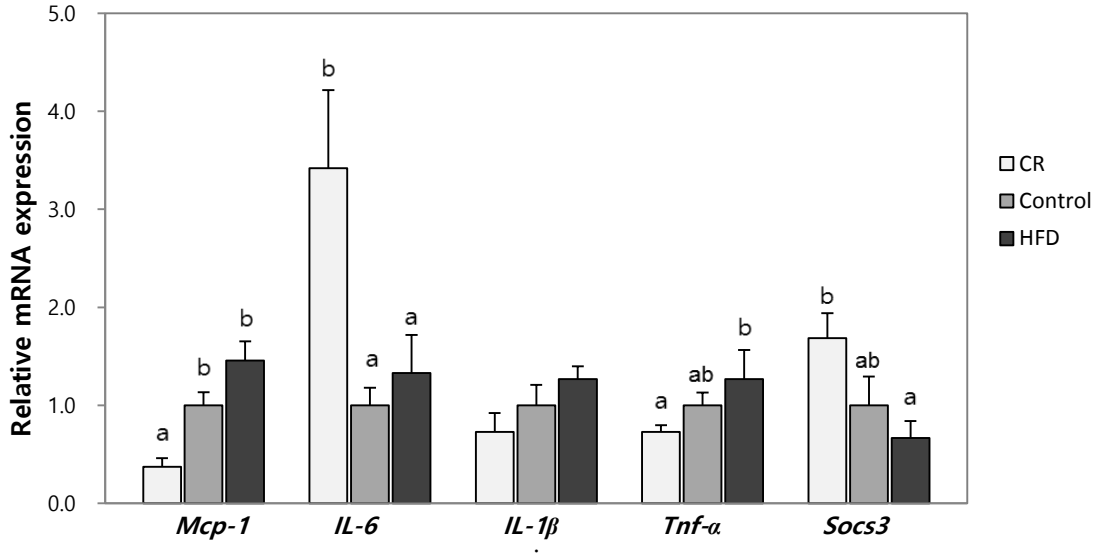
Hepatic mRNA levels of *Mcp-1*, a chemokine that attracts cytokines to promote inflammatory responses, were significantly different among groups ($P < 0.01$). CR group had significantly lower *Mcp-1* expression than control group ($P < 0.01$) and HFD group ($P < 0.01$). Also, *Tnf-α* level was lower in CR group than HFD group ($P < 0.05$). There were no significant differences in mRNA expression levels of *IL-1β* among groups. *Mcp-1* mRNA level showed positive correlation with serum glucose ($r = 0.602$, $P < 0.01$), liver TG ($r = 0.514$, $P < 0.01$) and liver cholesterol ($r = 0.617$, $P < 0.01$) levels. *IL-1β* mRNA level showed a negative correlation with adiponectin ($r = -0.552$, $P < 0.01$), and positive correlations with mRNA levels of *Adipor2* ($r = 0.465$, $P < 0.05$) and *Pparg* ($r = 0.545$, $P < 0.05$).

Hepatic *Socs3* expression level of CR group was significantly higher than that of HFD group ($P < 0.01$) and tended to be higher than that of control group ($P < 0.1$). Also CR group had significantly higher *IL-6* expression than control group ($P < 0.01$) and HFD group ($P < 0.05$). The *IL-6* mRNA

level correlated positively with serum NEFA level ($r = 0.807$, $P < 0.001$)**.** And *Socs3* mRNA level showed positive correlation with *IL-6* level ($r = 0.446$, $P < 0.05$) and negative correlations with body weight ($r = -0.554$, $P < 0.01$), white adipose tissue amount ($r = -0.528$, $P < 0.05$), liver TG level ($r = 0.572$, $P < 0.01$) liver cholesterol level ($r = 0.557$, $P < 0.01$), serum leptin level ($r = -0.595$, $P < 0.01$), and mRNA level of *Pparg* ($r = -0.433$, $P < 0.05$).

In epididymal adipose tissue, mRNA levels of genes related to inflammation were significantly different among groups. Epididymal *Mcp-1* mRNA level in CR group was lower than that in HFD ($P < 0.001$) and control groups ($P < 0.05$). Control group had significantly lower *Mcp-1* expression than HFD group ($P < 0.001$). CR group had significantly lower mRNA expression of *IL-6* than HFD group ($P < 0.001$) and tended to have lower expression than control group ($P = 0.057$). *Tnf-α* mRNA level was significantly lower in CR group compared with HFD group ($P < 0.001$). *IL-1β* mRNA level was significantly lower in CR group than HFD group. In contrast to hepatic *Socs3* expression pattern, epididymal *Socs3* level was significantly lower in CR group compared with HFD group ($P < 0.01$) and showed positive correlations with body weight ($r = 0.727$, $P < 0.001$), white adipose tissue amount ($r = 0.665$, $P < 0.01$), serum leptin level ($r = 0.673$, $P < 0.01$), and *Pparg* mRNA level ($r = 0.696$, $P < 0.001$) **(Figure 13).**

A



B

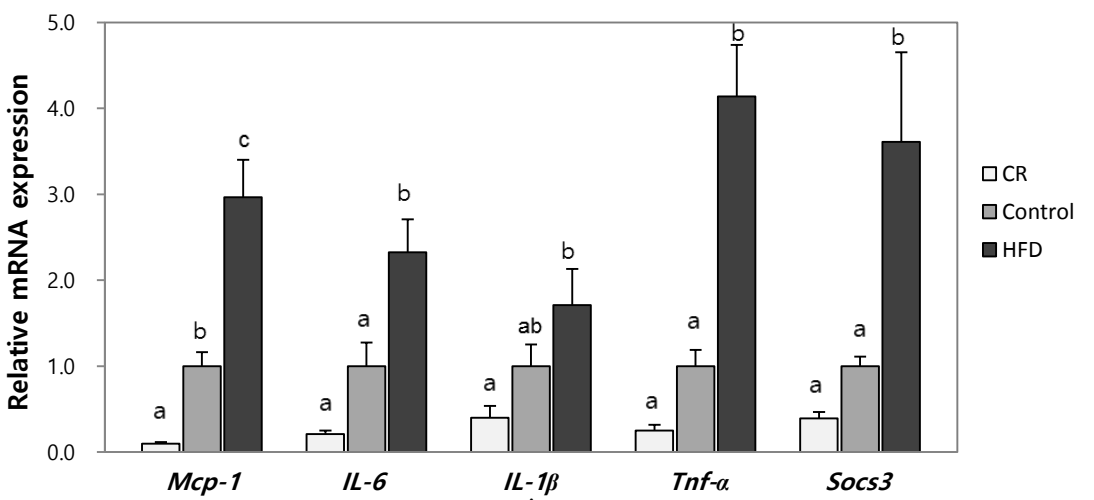


Figure 13. The mRNA levels of genes related to inflammation in liver and adipose tissue. (A) Relative mRNA expression levels of genes related to inflammation in liver, and (B) Relative mRNA expression levels of genes related to inflammation in epididymal adipose tissue

Data are presented as mean ± SE, n = 7 for each group. One-way ANOVA was used to determine the significant difference among groups. Different letters

indicate significant difference at $P < 0.05$ by Fisher's LSD multiple comparison test. All values were normalized to the levels of house-keeping gene *Gapdh* and expressed as relative mRNA level compared to the average level of Control group. *Mcp-1*, monocyte chemoattractant protein-1; *IL-6*, interleukin 6; *IL-1β*, interleukin 1beta; *Tnf-α*, tumor necrosis factor alpha; *Socs3*, suppressor of cytokine signaling 3.

8. Expression of genes involved in Socs3 transcription

In order to investigate the difference in *Socs3* mRNA expression pattern between liver and epididymal adipose tissue, we determined the expression of genes involved in leptin signaling pathway. Transcription of *Socs3* is related to down-stream signaling pathway of leptin **(Figure 16).**

Lepr, receptor of the leptin, mRNA level in liver was significantly higher in CR group than both Control and HFD groups. Hepatic *Lepr* mRNA level negatively correlated with serum leptin level ($r = -0.708$, $P < 0.001$), serum glucose level ($r = -0.717$, $P < 0.001$) and positively correlated with hepatic *Socs3* mRNA level ($r = 0.699$, $P < 0.001$) and *Ppara* mRNA level ($r = 0.712$, $P < 0.001$) **(Figure 14).** In epididymal adipose tissue, there was no significant difference in *Lepr* mRNA levels among groups. Epididymal adipose tissue *Lepr* mRNA level correlated with epididymal *IL-1β* mRNA level ($r = 0.437$, $P < 0.05$) but not with serum leptin level or epididymal *Socs3* mRNA level.

Upon activation, pSTAT3, phosphorylated form of STAT3, can induce *Socs3* expression. Levels of STAT3 and pSTAT3, and ratio of pSTAT3/STAT3 in liver did not differ among groups **(Figure 15).** pSTAT3 protein level and pSTAT3/STAT3 correlated with hepatic Socs3 mRNA level (pSTAT3, $r = 0.543$, $P < 0.05$; pSTAT3/STAT3, $r = 0.569$, $P < 0.01$).

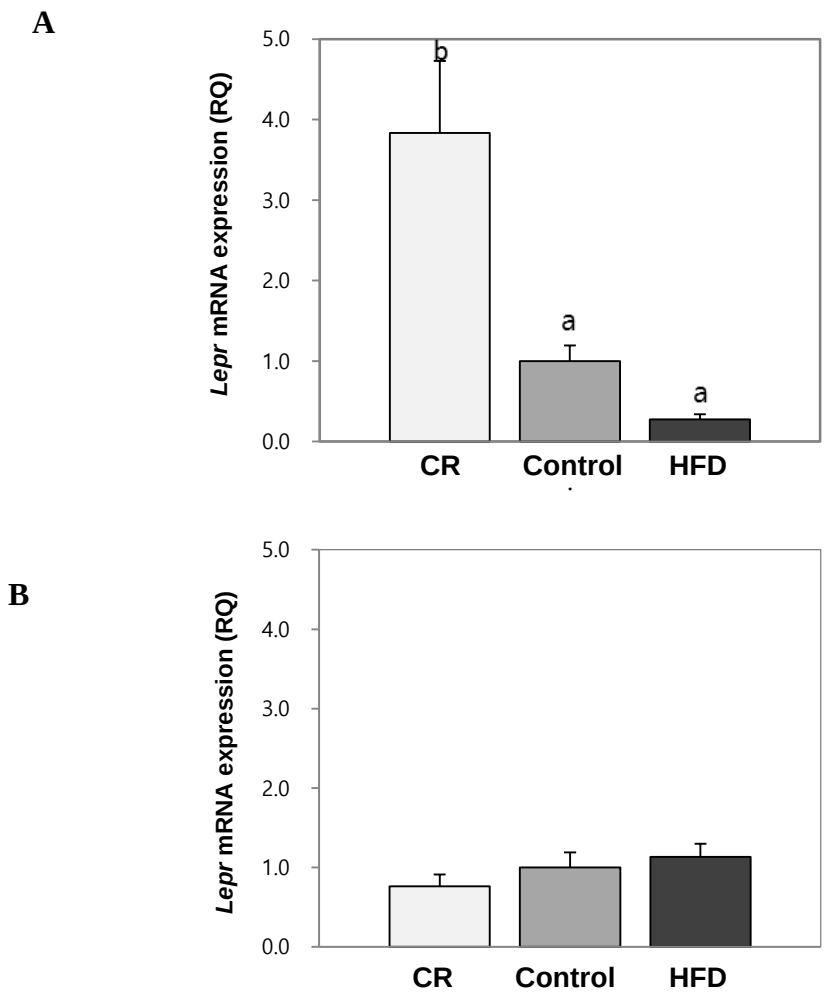


Figure 14. The mRNA levels of *Lepr* in liver and epididymal adipose tissue. **(A) Relative mRNA expression level of hepatic *Lepr*, and (B) Relative mRNA expression level of epididymal *Lepr***

Data are presented as mean ± SE, n = 7 for each group. One-way ANOVA was used to determine the significant difference among groups. Different letters indicate significant difference at $P < 0.05$ by Fisher's LSD multiple comparison test. All values were normalized to the levels of house-keeping gene *Gapdh* and expressed as relative mRNA level compared to the average level of Control group.

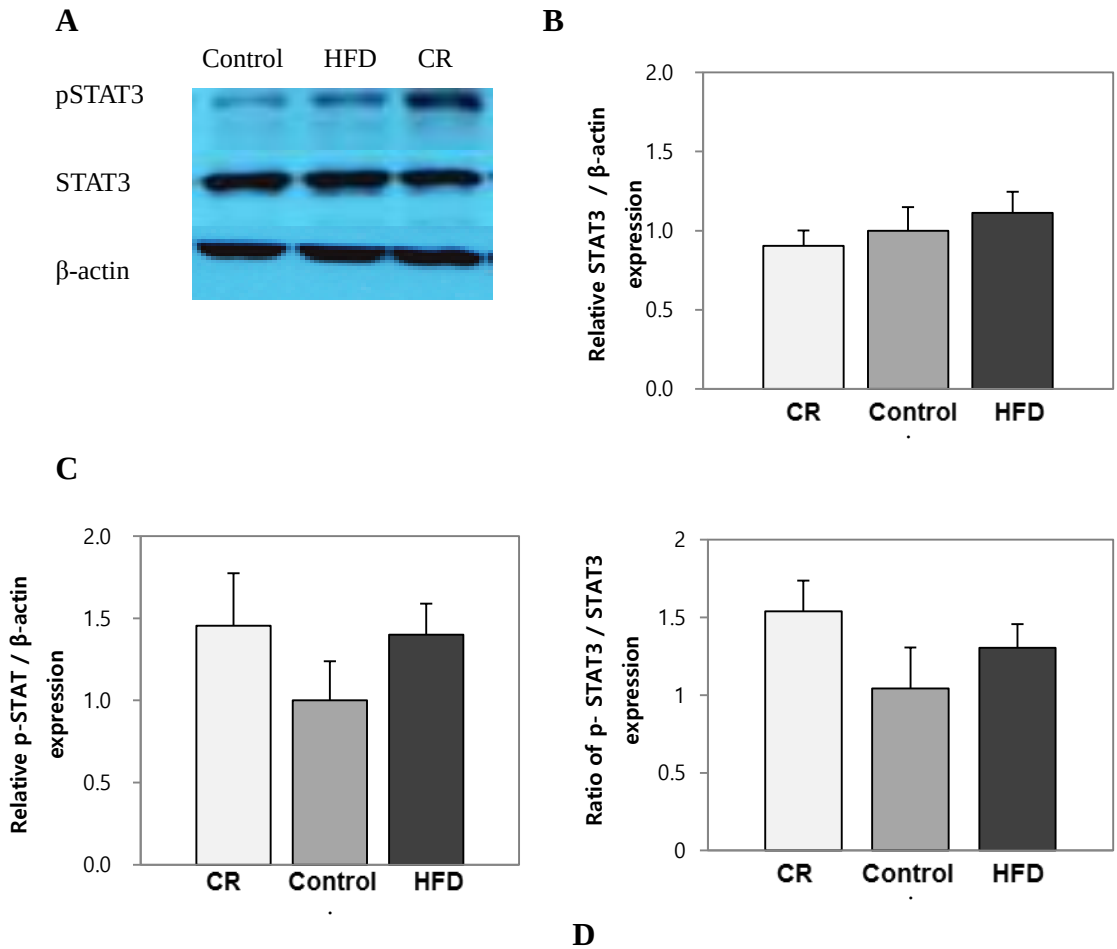


Figure 15. The protein expression levels of STAT3 and pSTAT3 in liver and hepatic STAT3 activation. (A) Western blotting for STAT3, pSTAT3, and β-actin (B) Densitometric analysis of STAT3 protein expression (C) Densitometric analysis of pSTAT3 protein expression, and (D) Ratio of pSTAT3/STAT3 expression

The intensity of pSTAT3 and STAT3 were densitometrically measured and normalized to the protein expression level of β-actin. Values are mean ± SE, n = 7 for each group. One-way ANOVA was used to determine the significant difference among groups.

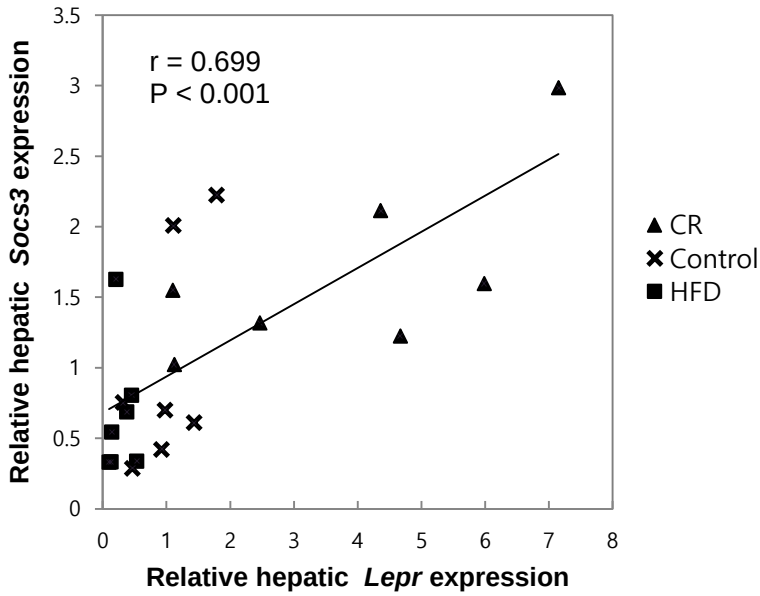


Figure 16. Correlation between hepatic *Lepr* mRNA levels and hepatic *Socs3* mRNA levels. Relation between hepatic *Lepr* level and hepatic *Socs*3 level. Pearson correlation coefficient, r, and P-value are indicated for each region. (n = 21).

Ⅴ. Discussion

In the present study, we investigated the effects of mild calorie restriction (14.1% energy reduction of *ad libitum* control group) on lipid metabolism and inflammatory response in liver and adipose tissue of mice. The CR group had lower body weight, white adipose tissue amount, and liver lipid contents and expressed lower level of genes related to inflammation in the liver and epididymal adipose tissue, except hepatic *IL-6* and *Socs3*. Although many previous studies showed advantages of calorie restriction related to obesity and non-alcoholic fatty liver diseases(NAFLD), they usually restricted more than 30% of *ad libitum* calories intake (Fan et al. 2003, Huang et al. 2010, Park et al. 2012). Therefore, we studied the realistic and achievable effects of mild calorie restriction on markers related to lipid metabolism and inflammation.

Serum fetuin-A level was significantly lower in CR group than other groups. Fetuin-A has been suggested as the hepatokine, which is up-regulated in response to increased liver fat mass and regulates metabolic syndrome such as NAFLD and type 2 diabetes mellitus (T2DM) (Haukeland et al. 2012, Kantartzis et al. 2010). Hennige et al. (2008) reported that fetuin-A promoted inflammatory cytokine expression and repressed production of adiponectin (Hennige et al. 2008, Stefan et al. 2013). Our data also showed that circulating fetuin-A level had positive correlations with

mRNA levels of hepatic *Mcp-1* and *Tnf-α* and epididymal *IL-6*, *IL-1β*, and *Tnf-α* (hepatic *Mcp-1*, r = 0.633, P= 0.002; hepatic *Tnf-α*, r = 0.493, P = 0.027; epididymal *IL-6*, r = 0.465, P = 0.034; epididymal *Tnf-α*, r = 0.657, P = 0.001), and had a negative correlation with serum adiponectin level (r = -0.452, P = 0.040). Unlike circulating fetuin-A level, hepatic mRNA levels of fetuin-A*(Ahsg*) were not different among groups, which is similar with the result by Haukeland et al. (Haukeland et al. 2012). Hepatic *Ahsg* mRNA level showed significantly positive correlations with hepatic mRNA levels of genes related to lipid metabolism (*Srebf-1c*, r = 0.564, P = 0.08; *Ppara*, r = 0.770, P < 0.001; *Cpt1a*, r = 0.744, P < 0.001). Haukeland et al. (2008) also reported that regulation of hepatic fetuin-A secretion is not well known, but there was a strong co-regulation between fetuin-A mRNA expression and lipid metabolism related genes. Therefore, in spite of no differences in hepatic mRNA levels of fetuin-A, we can speculate that calorie restriction could reduce serum fetuin-A level and attenuate the levels of inflammatory markers in liver and adipose tissue and could have relation with the level of genes related to lipid metabolism, such as *Cpt1a* and *Srebf-1c*.

We did not observe any differences in hepatic mRNA levels of *Adipor2*, *Cpt1a*, and *Ppara*, which are related to fatty acid oxidation. The protein SIRT1 level was not different among groups. Calorie restriction has been known to trigger SIRT1 activation (Canto et al. 2010). SIRT1 is necessary

for mediating the effects of calorie restriction because it can have an influence on the decrease in hepatic TG level (Schug et al. 2011). *Ppara* signaling is activated by SIRT1 as recent study showed that SIRT1 hepatic knock-out mice resulted in impaired *Ppara* signaling and decreased expression of genes involved in beta-oxidation (Purushotham et al. 2009). *Ppara* was identified as an up-regulator of *Cpt1a* enzyme that transfers long chain fatty acid into mitochondria for beta-oxidation. In our study, the mRNA expression of *Cpt1a* showed a positive correlation with *Ppara* ($r = 0.822$, $P < 0.001$). Our calorie restriction model limited only 14.1% of the energy compared with the control group, whereas recent studies which showed up-regulation of SIRT1 expression restricted calorie by 40% or even fasted for 6 days (Canto et al. 2010, Cohen et al. 2004, Pedersen et al. 2008). We speculated that SIRT1 expression was not significantly altered in our calorie restricted group because the magnitude of calorie restriction was mild therefore hepatic *Ppara* and *Cpt1a* levels were not up-regulated in CR group.

Hepatic parameters involved in lipid synthesis, *Srebf-1c* and *Pparg*, were significantly different among groups, and especially HFD group showed higher mRNA expressions than other groups. However, there was no further reduction in expression of these genes involved in lipid synthesis by calorie restriction as *Srebf-1c* and *Pparg* mRNA levels were not significantly

different between CR and Control groups. Although *Pparg* was reported to regulate lipid synthesis in adipocyte, previous studies reported an increased hepatic *Pparg* expression in fatty liver as well (Vidal-Puig et al. 1996). *Srebf-1c*, DNA binding transcription factor, is involved in the regulation of lipid metabolism as it activates lipogenic genes, such as *Fasn*, stearoyl-CoA desaturase-1(*SCA-1)*, and glyceraldehyde 3-phosphate acyltransferase (*GPAT*) (Denechaud et al. 2008, Horton 2002, Kemper et al. 2013). Horton et al. (1998) reported that hepatic nuclear *Srebf-1c* declined at fasting state and Ponougoti et al. (2010) found that activated SIRT1 protein could inhibit *Srebf-1c* transactivation. Therefore, SIRT1 activation could suppress the *Srebf-1c* expression through calorie restriction (Ponugoti et al. 2010). However, in the present study, mild calorie restriction did not affect the levels of *Srebf-1c* and SIRT1. In accordance with the study by Denechaud et al. (2008), *Fasn*, fatty acid synthesis enzyme, had positive correlation with *Srebf-1c* although mRNA expression of *Fasn* was not different among groups ($r = 0.444$, $P = 0.044$).

Significantly higher levels of pro-inflammatory cytokines and chemokine, including *IL-6, IL-1β, Tnf-α,* and *Mcp-1* mRNAs in epididymal fat tissue, were observed in HFD group compared with Control and CR groups. Expansion of the adipose tissue during weight gain resulted in macrophage infiltration into adipose tissue through various signals (Tilg et al. 2006).

Mcp-1 is secreted from adipose tissue and macrophage and it attracts monocytes or other macrophage. In our study, epididymal *Mcp-1* mRNA expression was significantly lower and epididymal *IL-6* mRNA expression tended to be lower in the CR group than the control group (*Mcp-1*, P = 0.029; *IL-6*, P = 0.057). However there were no differences in mRNA levels of *IL-1β* and *Tnf-α* between CR and control groups. According to the study by Kanda et al. (2006), macrophage infiltration into adipose tissue was increased in Mcp-1 transgenic mice, while decreased in Mcp-1 Knock-out mice, even when the same diet was fed to all groups (Kanda et al. 2006). Also, Mcp-1 secretion from adipose tissue might increase the expressions of hepatic *Srebf-1c, Pepck,* and *G6pase* by interacting with *Ccr2* in the liver (Kanda et al. 2006, Maher et al. 2008, Tilg et al. 2006). In our study, epididymal *Mcp-1* mRNA level positively correlated with hepatic *Srebf-1c* mRNA level (r = 0.584, P = 0.005), liver TG content (r = 0.647, P = 0.002), and liver cholesterol level (r = 0.683, P = 0.001). Therefore, it is possible that lower expression of epididymal *Mcp-1* with mild calorie restriction might have contributed to attenuate lipid synthesis in liver by decreasing *Srebf-1c* level.

In the liver, mRNA expressions of pro-inflammatory cytokines showed different patterns depending on the genes. The mRNA level of *Mcp-1* was significantly lower in CR group than Control (P = 0.022) and HFD groups

(P < 0.001) and *Tnf-α* level was significantly lower in CR group than HFD group (P = 0.011). On the other hand, mRNA expression of *IL-6* was higher in CR group than Control and HFD groups, and it was positively correlated with circulating NEFA level (r = 0.807, P < 0.001). Several recent studies have reported that Tnf-α is the crucial factor for the early phase of non-alcoholic steatohepatitis(NASH) development (Szabo et al. 2012, Tomita et al. 2006, Tosello-Trampont et al. 2012). Tnf-α is mainly produced by kupffer cells and it stimulated secretion of Mcp-1 by allowing the recruitment of blood monocyte in the mice model (Tosello-Trampont et al. 2012). In the present study, only CR group but not Control group showed significantly lower expression of *Tnf-α* level compared with HFD group. Thus, it suggested that HFD could trigger the liver lesion of NASH.

The reason why hepatic *IL-6* and *IL-1β* levels were not up-regulated in HFD group is thought to be that high fat diet (60% kcal fat) for 16 weeks was insufficient to induce severe phase of NASH. According to previous studies, diet-induced NASH mice models were generally fed methionine and choline deficient (MCD) diet for 10 days or HFD diet for very long period such as 60 weeks (Miura et al. 2012, Nakamura et al. 2013). Even though IL-1β is an inflammatory marker in liver, IL-1β is secreted mostly by activated macrophages and IL-1β plays a role in enhancing the cytotoxicity of Tnf-α (Szabo et al. 2012, Tosello-Trampont et al. 2012). It

seemed that there was no difference in *IL-1β* mRNA level among groups because liver of HFD group, fed HFD for 16 weeks, was just in the early stage of NASH (Nakamura et al. 2013).

In the present study, mRNA expressions of *Socs3* were significantly different among groups in both liver and adipose tissues, but, levels in two different tissues showed opposite direction among group. In the liver, *Socs3* level of CR group was significantly higher than that of HFD group, but in the adipose tissue, *Socs3* level was lower in CR group than HFD group. *Socs3* expression can be explained by chronic JAK-STAT-SOCS3 signaling regulated by leptin or cytokines (La Cava et al. 2004, Wunderlich et al. 2013). Higher liver *Socs3* level in CR group might be explained by increased peripheral leptin sensitivity and up-regulated hepatic *IL-6* level. In the liver, mRNA level of *Lepr*, receptor for the circulating leptin, was significantly up-regulated in CR group and hepatic *Lepr* level was positively correlated with hepatic *Socs*3 mRNA level ($r = 0.699$, $P < 0.001$). Hepatic *IL-6* level was higher in CR group than other groups and had positive correlation with hepatic *Socs3* expression level ($r = 0.446$, $P < 0.049$). Senn et al. (2003) also reported that hepatic *Socs3* expression was induced in the liver when mice were injected with IL-6. Hepatic phosphorylation of STAT3, one of the potential mediator of *Socs3* transcription, was positively correlated with hepatic *Socs3* expression ($r = 0.543$, $P = 0.011$), hepatic *IL-6*

expression (r = 0.596, P = 0.004), and had tendency of positive correlation with *Lepr* (r = 0.432, P = 0.051). Kang et al (2013) also reported that calorie restriction and exercise increased *Socs3* and *Lepr* expression in liver, resulted in improved peripheral leptin resistance (Kang et al. 2013). In many recent studies, up-regulated hepatic *Socs3* led to insulin resistance by inhibiting phosphorylation of IRS-1, which is the important intermediate of the insulin signaling (Wunderlich et al. 2013). Higher expression of hepatic *Socs3* in CR group could cause insulin resistance. However insulin resistance during lower energy state, but not with surplus fat, could play beneficial role to spare glucose by suppressing hepatic glycogen synthesis and glycolysis (Kang et al. 2013, Tsatsoulis et al. 2013). In current study, serum glucose level was significantly lower in CR group than other groups, hepatic *Lepr* showed significantly negative correlation with glucose level, and hepatic *IL-6* tended to correlate negatively with serum glucose level (*Lepr*, r = -0.717, P < 0.001; *IL-6*, r = -0.419, P = 0.066). Therefore it is suggested that insulin resistance caused by up-regulated hepatic *Socs3, IL-6* and *Lepr* might have reduced blood glucose consumption by liver.

Higher epididymal *Socs3* level in HFD group might be explained by cytokine responses. Contrary to liver, adipose *Lepr* were not different among groups, and did not have correlation with adipose *Socs3* level, whereas epididymal *IL-6* and *Tnf-α* levels correlated positively with

epididymal *Socs3* level (*IL-6*, r = 0.620, P < 0.001; *Tnf-α*, r = 0.672, P < 0.001). Socs3 was shown to be up-regulated in response to Tnf-α expression in the adipose tissue (Emanuelli et al. 2001). Collectively, it is thought that hepatic leptin sensitivity was improved and hepatic *IL-6* and *Socs3* were up-regulated by CR to spare serum glucose. And in adipose tissue, attenuated inflammatory markers such as *IL-6* and *Tnf-α* contributed to reduced *Socs3* expression.

In conclusion, the mRNA levels of genes related to hepatic lipid metabolism, such as *Ppara, Srebf-1c* were not additionally affected by mild CR, since mild CR did not up-regulate hepatic SIRT1 protein level which has been known to regulate these genes. The low mRNA levels of epididymal *Mcp-1* and *IL-6* in CR group suggested that mild calorie restriction could ameliorate macrophage infiltration into adipose tissue. Also, mild calorie restriction alleviated hepatic inflammatory markers by reducing mRNA levels of *Mcp-1* and *Tnf-α*. However, because of low serum glucose level in CR group, hepatic *Socs3* was up-regulated through the high expression of hepatic *IL-6* and *Lepr* in the liver. As a result mild calorie restriction could reduce hepatic lipid level, decrease expression of markers related to inflammation in liver and adipose tissues, and besides it might have caused beneficial insulin resistance in order to sparing blood glucose.

VI. SUMMARY

In this study, the effects of mild calorie restriction, compared with Control and high-fat diets, on the markers related to lipid metabolism and inflammation were investigated. After feeding mice for 16 weeks with control diet containing 10% kcal fat (Control) or high fat diet containing 60% kcal fat (HFD) or reduced amount of control diet to achieve 14.1% calorie restriction (CR), body weight, the amount of white adipose tissue, serum adipokines, TG, cholesterol, NEFA, glucose and the expression of genes involved in lipid metabolism and inflammation were measured. The results of the present study were as follows:

1) CR group had significantly lower body weight gain, liver weight, and liver lipid level than Control and HFD groups. CR group had significantly lower serum leptin level, higher adiponectin level than Control and HFD groups. And serum fetuin-A level was significantly lower in CR group than other groups.

2) Serum TG level was significantly lower in CR group than HFD group and serum cholesterol and glucose levels were significantly lower than Control and HFD groups. However serum NEFA level was significantly higher in CR group than the other groups.

3) There was no significant difference in mRNA level of genes involved in fatty acid oxidation in liver. Also the protein level of SIRT1 was not different among groups. The mRNA levels of genes related to lipid synthesis, *Srebf-1c and Pparg*, were significantly lower in CR group compared with HFD group, whereas not lower than Control group.

4) CR group had significantly lower mRNA level of *Mcp-1* than Control group, and had the tendency of lower *IL-6* mRNA level in adipose tissue compared with Control group. Also mRNA levels of *Tnf-α, IL-1β*, and *Socs3* were lower in CR group than HFD group, but not lower than Control group.

5) CR group had significantly lower mRNA levels of hepatic *Mcp-1* and *Tnf-α* in CR group compared with HFD group. On the contrary, hepatic *IL-6* and *Socs3* mRNA levels were significantly higher in CR group than HFD group.

6) Hepatic *Lepr* mRNA level in liver was significantly higher in CR group, than other groups, and had positive correlation with hepatic *Socs3* level. Also, ratio of pSTAT3/STAT3 had positive correlations with hepatic *IL-6* and *Socs3* levels.

These results indicate that mild calorie restriction not only reduced weight gain, amount of white adipose tissue and hepatic lipid level but also reduced

expressions of inflammatory markers in liver and adipose tissue. The low mRNA expression levels of epididymal *Mcp-1* and *IL-6* in CR group and hepatic *Mcp-1* and *Tnf-α* suggested that mild CR could attenuate chronic inflammatory response. However, increased hepatic *IL-6* and improved leptin sensitivity via CR might have up-regulated hepatic *Socs3*expression, resulted in beneficial hepatic insulin resistance in order to sparing serum glucose.

Ⅶ. References

Aguirre V, Uchida T, Yenush L, Davis R, White MF. "The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307)." J Biol Chem 2000, 275(12): 9047-9054.

Alba LM, Lindor K. "Review article: Non-alcoholic fatty liver disease." Aliment Pharmacol Ther 2003, 17(8): 977-986.

Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, Wynshaw-Boris A, Poli G, Olefsky J, Karin M. "IKK-beta links inflammation to obesity-induced insulin resistance." Nat Med 2005, 11(2): 191-198.

Azhar S. "Peroxisome proliferator-activated receptors, metabolic syndrome and cardiovascular disease." Future Cardiol 2010, 6(5): 657-691.

Bastard JP, Hainque B, Dusserre E, Bruckert E, Robin D, Vallier P, Perche S, Robin P, Turpin G, Jardel C, Laville M, Forest C, Vidal H. "Peroxisome proliferator activated receptor-gamma, leptin and tumor necrosis factor-alpha mRNA expression during very low calorie diet in subcutaneous adipose tissue in obese women." Diabetes Metab Res Rev 1999, 15(2): 92-98.

Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, Vidal H, Hainque B. "Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss." J Clin Endocrinol Metab 2000, 85(9): 3338-3342.

Bechmann LP, Hannivoort RA, Gerken G, Hotamisligil GS, Trauner M, Canbay A. "The interaction of hepatic lipid and glucose metabolism in liver diseases." J Hepatol 2012, 56(4): 952-964.

Bjorbaek C, El-Haschimi K, Frantz JD, Flier JS. "The role of SOCS-3 in leptin signaling and leptin resistance." J Biol Chem 1999, 274(42): 30059-30065.

Bragt MC, Popeijus HE. "Peroxisome proliferator-activated receptors and the metabolic syndrome." Physiol Behav 2008, 94(2): 187-197.

Buckman LB, Hasty AH, Flaherty DK, Buckman CT, Thompson MM, Matlock BK, Weller K, Ellacott KL. "Obesity induced by a high-fat diet is associated with increased immune cell entry into the central nervous system." Brain Behav Immun 2013,

Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE. "Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB." Nat Med 2005, 11(2): 183-190.

Canto C, Jiang LQ, Deshmukh AS, Mataki C, Coste A, Lagouge M, Zierath JR, Auwerx J. "Interdependence of AMPK and SIRT1 for metabolic adaptation to fasting and exercise in skeletal muscle." Cell Metab 2010, 11(3): 213-219.

Carmiel-Haggai M, Cederbaum AI, Nieto N. "A high-fat diet leads to the progression of non-alcoholic fatty liver disease in obese rats." FASEB J 2005, 19(1): 136-138.

Cerqueira FM, Kowaltowski AJ. "Commonly adopted caloric restriction protocols often involve malnutrition." Ageing Res Rev 2010, 9(4): 424-430.

Chakravarthy MV, Pan Z, Zhu Y, Tordjman K, Schneider JG, Coleman T, Turk J, Semenkovich CF. ""New" hepatic fat activates PPARalpha to maintain glucose, lipid, and cholesterol homeostasis." Cell Metab 2005, 1(5): 309-322.

Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA. "Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase." Science 2004, 305(5682): 390-392.

Denechaud PD, Bossard P, Lobaccaro JM, Millatt L, Staels B, Girard J, Postic C. "ChREBP, but not LXRs, is required for the induction of glucose-regulated genes in mouse liver." J Clin Invest 2008, 118(3): 956-964.

Desvergne B, Wahli W. "Peroxisome proliferator-activated receptors: nuclear control of metabolism." Endocr Rev 1999, 20(5): 649-688.

Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ, Hotamisligil GS, Van Obberghen E. "SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice." J Biol Chem 2001, 276(51): 47944-47949.

Fabbrini E, Sullivan S, Klein S. "Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications." Hepatology 2010, 51(2): 679-689.

Fan JG, Zhong L, Xu ZJ, Tia LY, Ding XD, Li MS, Wang GL. "Effects of low-calorie diet on steatohepatitis in rats with obesity and hyperlipidemia." World J Gastroenterol 2003, 9(9): 2045-2049.

Fantuzzi G. "Adipose tissue, adipokines, and inflammation." J Allergy Clin Immunol 2005, 115(5): 911-919; quiz 920.

Ferre P, Foufelle F. "Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c." Diabetes Obes Metab 2010, 12 Suppl 2(83-92).

Folch J, Lees M, Sloane Stanley GH. "A simple method for the isolation and purification of total lipides from animal tissues." J Biol Chem 1957, 226(1):

497-509.

Foretz M, Guichard C, Ferre P, Foufelle F. "Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes." Proc Natl Acad Sci U S A 1999, 96(22): 12737-12742.

Gerner RR, Wieser V, Moschen AR, Tilg H. "Metabolic inflammation: role of cytokines in the crosstalk between adipose tissue and liver." Can J Physiol Pharmacol 2013, 91(11): 867-872.

Gobin S, Thuillier L, Jogl G, Faye A, Tong L, Chi M, Bonnefont JP, Girard J, Prip-Buus C. "Functional and structural basis of carnitine palmitoyltransferase 1A deficiency." J Biol Chem 2003, 278(50): 50428-50434.

Gregor MF, Hotamisligil GS. "Inflammatory mechanisms in obesity." Annu Rev Immunol 2011, 29(415-445.

Grunfeld C, Adi S, Soued M, Moser A, Fiers W, Feingold KR. "Search for mediators of the lipogenic effects of tumor necrosis factor: potential role for interleukin 6." Cancer Res 1990, 50(14): 4233-4238.

Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. "Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in

low density lipoprotein receptor-deficient mice." Mol Cell 1998, 2(2): 275-281.

Haukeland JW, Dahl TB, Yndestad A, Gladhaug IP, Loberg EM, Haaland T, Konopski Z, Wium C, Aasheim ET, Johansen OE, Aukrust P, Halvorsen B, Birkeland KI. "Fetuin A in nonalcoholic fatty liver disease: in vivo and in vitro studies." Eur J Endocrinol 2012, 166(3): 503-510.

Hennige AM, Staiger H, Wicke C, Machicao F, Fritsche A, Haring HU, Stefan N. "Fetuin-A induces cytokine expression and suppresses adiponectin production." PLoS One 2008, 3(3): e1765.

Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. "A central role for JNK in obesity and insulin resistance." Nature 2002, 420(6913): 333-336.

Horton JD. "Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis." Biochem Soc Trans 2002, 30(Pt 6): 1091-1095.

Horton JD, Bashmakov Y, Shimomura I, Shimano H. "Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice." Proc Natl Acad Sci U S A 1998, 95(11): 5987-5992.

Hotamisligil GS. "Inflammation and metabolic disorders." Nature 2006, 444(7121): 860-867.

Hotamisligil GS, Shargill NS, Spiegelman BM. "Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance." Science 1993, 259(5091): 87-91.

Huang P, Li S, Shao M, Qi Q, Zhao F, You J, Mao T, Li W, Yan Z, Liu Y. "Calorie restriction and endurance exercise share potent anti-inflammatory function in adipose tissues in ameliorating diet-induced obesity and insulin resistance in mice." Nutr Metab (Lond) 2010, 7(59).

Iizuka K, Bruick RK, Liang G, Horton JD, Uyeda K. "Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as well as glycolysis." Proc Natl Acad Sci U S A 2004, 101(19): 7281-7286.

Jensen-Urstad AP, Semenkovich CF. "Fatty acid synthase and liver triglyceride metabolism: housekeeper or messenger?" Biochim Biophys Acta 2012, 1821(5): 747-753.

Kahn SE, Hull RL, Utzschneider KM. "Mechanisms linking obesity to insulin resistance and type 2 diabetes." Nature 2006, 444(7121): 840-846.

Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M. "MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity." J Clin Invest 2006, 116(6): 1494-1505.

Kang S, Kim KB, Shin KO. "Exercise training improves leptin sensitivity in peripheral tissue of obese rats." Biochem Biophys Res Commun 2013, 435(3): 454-459.

Kantartzis K, Machann J, Schick F, Fritsche A, Haring HU, Stefan N. "The impact of liver fat vs visceral fat in determining categories of prediabetes." Diabetologia 2010, 53(5): 882-889.

Kaplan LM. "Leptin, obesity, and liver disease." Gastroenterology 1998, 115(4): 997-1001.

Kemper JK, Choi SE, Kim DH. "Sirtuin 1 deacetylase: a key regulator of hepatic lipid metabolism." Vitam Horm 2013, 91(385-404.

Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB. "The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase." J Clin Invest 1995, 95(5): 2111-2119.

Koo SH. "Nonalcoholic fatty liver disease: molecular mechanisms for the hepatic steatosis." Clin Mol Hepatol 2013, 19(3): 210-215.

La Cava A, Matarese G. "The weight of leptin in immunity." Nat Rev Immunol 2004, 4(5): 371-379.

Li X, Cope MB, Johnson MS, Smith DL, Jr., Nagy TR. "Mild calorie restriction induces fat accumulation in female C57BL/6J mice." Obesity (Silver Spring) 2010, 18(3): 456-462.

Liu Q, Bengmark S, Qu S. "The role of hepatic fat accumulation in pathogenesis of non-alcoholic fatty liver disease (NAFLD)." Lipids Health Dis 2010, 9(42.

Maher JJ, Leon P, Ryan JC. "Beyond insulin resistance: Innate immunity in nonalcoholic steatohepatitis." Hepatology 2008, 48(2): 670-678.

Mathieu P, Lemieux I, Despres JP. "Obesity, inflammation, and cardiovascular risk." Clin Pharmacol Ther 2010, 87(4): 407-416.

McCay CM, Crowell MF, Maynard LA. "The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935." Nutrition 1989, 5(3): 155-171; discussion 172.

Miura K, Yang L, van Rooijen N, Ohnishi H, Seki E. "Hepatic recruitment of macrophages promotes nonalcoholic steatohepatitis through CCR2." Am J Physiol Gastrointest Liver Physiol 2012, 302(11): G1310-1321.

Musso G, Gambino R, Cassader M. "Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD)." Prog Lipid Res 2009, 48(1): 1-26.

Nakamura A, Terauchi Y. "Lessons from Mouse Models of High-Fat Diet-Induced NAFLD." Int J Mol Sci 2013, 14(11): 21240-21257.

Nteeba J, Ortinau LC, Perfield JW, 2nd, Keating AF. "Diet-induced obesity alters immune cell infiltration and expression of inflammatory cytokine genes in mouse ovarian and peri-ovarian adipose depot tissues." Mol Reprod Dev 2013,

Palanivel R, Fullerton MD, Galic S, Honeyman J, Hewitt KA, Jorgensen SB, Steinberg GR. "Reduced Socs3 expression in adipose tissue protects female mice against obesity-induced insulin resistance." Diabetologia 2012, 55(11): 3083-3093.

Pardina E, Baena-Fustegueras JA, Catalan R, Galard R, Lecube A, Fort JM, Allende H, Vargas V, Peinado-Onsurbe J. "Increased expression and activity of hepatic lipase in the liver of morbidly obese adult patients in relation to lipid content." Obes Surg 2009, 19(7): 894-904.

Park S, Park NY, Valacchi G, Lim Y. "Calorie restriction with a high-fat diet effectively attenuated inflammatory response and oxidative stress-related markers in obese tissues of the high diet fed rats." Mediators Inflamm 2012, 2012(984643.

Ponugoti B, Kim DH, Xiao Z, Smith Z, Miao J, Zang M, Wu SY, Chiang CM, Veenstra TD, Kemper JK. "SIRT1 deacetylates and inhibits SREBP-1C

activity in regulation of hepatic lipid metabolism." J Biol Chem 2010, 285(44): 33959-33970.

Purushotham A, Schug TT, Xu Q, Surapureddi S, Guo X, Li X. "Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation." Cell Metab 2009, 9(4): 327-338.

Salas-Salvado J, Bullo M, Garcia-Lorda P, Figueredo R, Del Castillo D, Bonada A, Balanza R. "Subcutaneous adipose tissue cytokine production is not responsible for the restoration of systemic inflammation markers during weight loss." Int J Obes (Lond) 2006, 30(12): 1714-1720.

Schug TT, Li X. "Sirtuin 1 in lipid metabolism and obesity." Ann Med 2011, 43(3): 198-211.

Senn JJ, Klover PJ, Nowak IA, Zimmers TA, Koniaris LG, Furlanetto RW, Mooney RA. "Suppressor of cytokine signaling-3 (SOCS-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes." J Biol Chem 2003, 278(16): 13740-13746.

Shimomura I, Bashmakov Y, Horton JD. "Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus." J Biol Chem 1999, 274(42): 30028-30032.

Soeters MR, Soeters PB. "The evolutionary benefit of insulin resistance." Clin Nutr 2012, 31(6): 1002-1007.

Song MJ, Kim KH, Yoon JM, Kim JB. "Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes." Biochem Biophys Res Commun 2006, 346(3): 739-745.

Spindler SR. "Caloric restriction: from soup to nuts." Ageing Res Rev 2010, 9(3): 324-353.

Stefan N, Haring HU. "The role of hepatokines in metabolism." Nat Rev Endocrinol 2013, 9(3): 144-152.

Szabo G, Csak T. "Inflammasomes in liver diseases." J Hepatol 2012, 57(3): 642-654.

Tauriainen E, Luostarinen M, Martonen E, Finckenberg P, Kovalainen M, Huotari A, Herzig KH, Lecklin A, Mervaala E. "Distinct effects of calorie restriction and resveratrol on diet-induced obesity and Fatty liver formation." J Nutr Metab 2011, 2011(525094.

Tilg H, Diehl AM. "Cytokines in alcoholic and nonalcoholic steatohepatitis." N Engl J Med 2000, 343(20): 1467-1476.

Tilg H, Moschen AR. "Adipocytokines: mediators linking adipose tissue, inflammation and immunity." Nat Rev Immunol 2006, 6(10): 772-783.

Tomita K, Tamiya G, Ando S, Ohsumi K, Chiyo T, Mizutani A, Kitamura N, Toda K, Kaneko T, Horie Y, Han JY, Kato S, Shimoda M, Oike Y, Tomizawa M, Makino S, Ohkura T, Saito H, Kumagai N, Nagata H, Ishii H, Hibi T. "Tumour necrosis factor alpha signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice." Gut 2006, 55(3): 415-424.

Tosello-Trampont AC, Landes SG, Nguyen V, Novobrantseva TI, Hahn YS. "Kuppfer cells trigger nonalcoholic steatohepatitis development in diet-induced mouse model through tumor necrosis factor-alpha production." J Biol Chem 2012, 287(48): 40161-40172.

Tsatsoulis A, Mantzaris MD, Bellou S, Andrikoula M. "Insulin resistance: an adaptive mechanism becomes maladaptive in the current environment - an evolutionary perspective." Metabolism 2013, 62(5): 622-633.

Ueki K, Kondo T, Tseng YH, Kahn CR. "Central role of suppressors of cytokine signaling proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse." Proc Natl Acad Sci U S A 2004, 101(28): 10422-10427.

Valdecantos MP, Perez-Matute P, Gonzalez-Muniesa P, Prieto-Hontoria PL, Moreno-Aliaga MJ, Martinez JA. "Lipoic acid administration prevents nonalcoholic steatosis linked to long-term high-fat feeding by modulating

mitochondrial function." J Nutr Biochem 2012, 23(12): 1676-1684.

Vidal-Puig A, Jimenez-Linan M, Lowell BB, Hamann A, Hu E, Spiegelman B, Flier JS, Moller DE. "Regulation of PPAR gamma gene expression by nutrition and obesity in rodents." J Clin Invest 1996, 97(11): 2553-2561.

Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante AW, Jr. "CCR2 modulates inflammatory and metabolic effects of high-fat feeding." J Clin Invest 2006, 116(1): 115-124.

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. "Obesity is associated with macrophage accumulation in adipose tissue." J Clin Invest 2003, 112(12): 1796-1808.

Wunderlich CM, Hovelmeyer N, Wunderlich FT. "Mechanisms of chronic JAK-STAT3-SOCS3 signaling in obesity." Jakstat 2013, 2(2): e23878.

Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. "Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance." J Clin Invest 2003, 112(12): 1821-1830.

Xydakis AM, Case CC, Jones PH, Hoogeveen RC, Liu MY, Smith EO, Nelson KW, Ballantyne CM. "Adiponectin, inflammation, and the expression of the metabolic syndrome in obese individuals: the impact of

rapid weight loss through caloric restriction." J Clin Endocrinol Metab 2004, 89(6): 2697-2703.

Yu BP. "Why calorie restriction would work for human longevity." Biogerontology 2006, 7(3): 179-182.

국문초록

경정도의 열량제한이 간과 지방조직의 지질대사와 염증반응에 미치는 영향

서울대학교 대학원 식품영양학과

박 찬 윤

비만은 세계적으로 증가 추세이며, 특히 고지방으로 유도된 비만은 인슐린 저항증, 비알콜성 지방간 등의 대사질환과 밀접한 관계가 있다. 비만으로 인해 지방세포가 팽창하면, 대식세포가 지방조직으로의 침윤되어 염증성 사이토카인이 분비되고 이는 지방조직에서의 염증반응을 야기한다. 이는 간으로 전달되어 간 조직 내부의 대식세포로부터 분비되는 염증성 물질들과 함께 지방간염을 유발하기도 한다. 열량제한은 지방과 간 조직에서의 지방세포의 팽창을 억제하여 지질대사를 개선할 뿐만 아니라, 이로 인한 염증 반응을 감소시킨다고 보고되어 왔다. 하지만 대부분의 동물 모델에서의 열량제한 실험은 대조군 식이의 30% 이상의 열량을 감소하였으며 이는 영양 부족을 야기하지는 않지만 사람에게 적용하기에는 현실성이 부족하다. 따라서 본 연구에서는 14.1%의 열량제한 모델을 대조군 그리고 고지방 식이로 유도한 비만군과 비교하여, 경정도의 열량제한이 간에서의 지질대사와

염증 반응에 미치는 영향을 알아보고 그 기전을 규명하고자 하였다. 7 주령의 수컷 C57BL/6 mice 를 세 군으로 나눈 후 두 가지 실험식이를 각각 16 주간 제공하였다. 각 군은 총 식이 열량의 10%을 지방으로 공급하는 저지방식이를 자유 급여하는 대조군, 총 식이 열량의 60%을 지방으로 공급하는 고지방식이를 자유 급여하는 고지방식이군, 그리고 고지방식이군이 섭취한 양을 저지방식이로 섭취하는 열량제한군으로 나뉘었다. 고지방식이군의 경우 자유 급여 시에도 섭취량은 감소하므로, 열량제한군은 대조군에 비하여 14.1%의 섭취 열량을 제한하였다. 혈청 leptin, adiponectin, fetuin-A, glucose 그리고 지질의 농도를 ELISA 와 비색 효소법을 이용하여 측정하였다. 간에서 지질 대사와 관련된 유전자인 *Pparg, Ppara, Fasn, Srebf-1c, Adipor2,* and *Cpt1a* 를, 간과 지방 조직에서 염증 반응 관련 유전자인 *IL-1β, IL-6, Mcp-1, Tnf-α,* 그리고 *Socs3* 의 mRNA 발현량을 Real-time PCR 로 측정하였다. 또한 간 조직에서 SIRT1, pSTAT3/STAT3 의 단백질 발현량을 Western blot 으로 측정하였다. 전체적으로 열량제한군에서 대조군과 고지방식이군에 비해 체중 증가량과 백색 지방량, 혈중 Cholesterol 그리고 Glucose 농도가 낮았다. 간에서 지방 합성과 관계된 *Pparg* 와 *Srebf-1c* 의 발현량은 열량제한군과 대조군이 고지방식이군에 비해 유의적으로 낮았다. 혈중 유리지방산 농도는 열량제한군이 다른 군들에 비해 유의적으로 높았다 ($P = 0.011$, $P = 0.007$). 부고환 지방 조직에서는 열량제한군에서의 *Mcp-1* 발현량이 대조군에 비해 유의적으로 낮았고 ($P = 0.027$), *IL-6* 발현량은 대조군보다 낮은 경향성을 보였다 ($P = 0.057$). 그 외

부고환 지방조직에서의 *IL-1β*, *Tnf-α* 그리고 *Socs3* 의 발현량은 열량제한군이 고지방식이군에 비하여 유의적으로 낮았으나, 대조군과는 유의적인 차이가 없었다. 다음으로 간에서는 *Mcp-1* 와 *Tnf-α* 발현량이 열량제한군에서 고지방식이군에 비하여 유의적으로 낮았다 (*Mcp-1,* 74% lower; *Tnf-α,* 47% lower). 하지만 그와는 반대로, 간에서의 *IL-6* 와 *Socs3* 발현량은 오히려 열량제한군이 고지방식이군에 비해 유의적으로 높았다. 열량제한군의 간에서 높은 *Socs3* 발현량은 낮은 혈중 glucose 로 인하여 야기된, *IL-6* 와 leptin 반응을 통한 STAT3 신호전달로 설명할 수 있다. 열량제한군에서의 렙틴 농도가 다른 두 군에 비해 유의적으로 낮았음에도 불구하고, 간에서의 *Lepr* 발현량은 열량제한군이 나머지 두 군에 비해 유의적으로 높았다. 간에서의 *IL-6* 발현량은 간에서의 *Socs3* 발현량(r= 0.446, P= 0.049), 간에서의 pSTAT3/STAT3 (r= 0.476, P= 0.034)와 유의적인 양의 상관관계를 혈중 glucose 농도와는 음의 상관관계의 경향성을 보였다 (r = −0.419, P = 0.066. 또한 간에서의 *Lepr* 발현량 역시 간에서의 *Socs3* 발현량 (r = 0.699, P< 0.001)과는 양의 상관관계를, 혈중 Glucose 농도와는 음의 상관관계를(r = −0.717, P < 0.001), 그리고 간에서의 pSTAT3/STAT3 와는 양의 상관관계의 경향성을 보였다(r = 0.432, P = 0.051). 결론적으로 본 연구는 경정도의 열량제한이 체중 증가량, 백색 지방량, 그리고 간에서의 지질농도를 감소시킴은 물론이고 지방조직과 간 조직에서의 염증반응을 완화시킬 수 있음을 시사한다. 단 경정도의 열량제한 시, 간에서 *IL-6* 발현이 증가되고 leptin 민감도가 증가하여, 간에서의 *Socs3* 발현이

증가하였는데, 이는 인슐린 저항증을 야기함으로 낮아진 혈중 glucose 농도를 유지시키기 위한 기작으로 사료된다.

주요어: 경정도의 열량제한, 간 지질대사, 간 염증반응, 지방조직 염증반응, *Socs3* 전사, leptin 민감도, 인슐린 저항증

학번: 2012-21491